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Targeting Hsp70 in Huntington's disease

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ABSTRACT

ABSTRACT

Polyglutamine disorders are a group of neurodegenerative diseases that result from expansions of CAG repeats in the affected genes with the ability to encode expanded glutamine stretches in the corresponding proteins, which render the protein highly prone to misfolding. The expanded polyglutamine protein may mediate pathogenesis through a range of mechanisms that include transcriptional dysregulation, mitochondrial dysfunction and abnormal proteostasis. Huntington's disease (HD) is a polyglutamine disorder in which the affected protein is huntingtin (Htt). Abnormal proteostasis in HD is associated with the accumulation and aggregation of mutant Htt (mHtt). The accumulation of misfolded mHtt is thought to overwhelm the chaperone machinery, consequently diverting an excess of other misfolded proteins to the degradation pathways and leading to a global collapse of the proteostasis network. Mitochondrial bioenergetics, dynamics and quality control are also disturbed in HD. mHtt may exert its effects on mitochondria indirectly or by direct interaction with the organelle and associated proteins. The chaperone machinery plays an essential role in the proteostasis network, acting in protein folding, trafficking, disaggregation and degradation. In response to a proteotoxic insult, such as the accumulation of misfolded proteins, the expression of molecular chaperones, known by heat shock proteins (Hsp), can be induced through activation of the heat shock response (HSR). However, the levels of different Hsp are reduced in several models of polyglutamine disorders. Results on genetic and pharmacological modulation of components of the molecular chaperone machinery support Hsp modulation as a potential therapeutic approach in polyglutamine disorders. Nevertheless, modulation of specific Hsp may exert different effects depending on the nature of the client protein.

A better understanding of how mHtt interferes with the chaperone machinery and, in turn, how Hsp modulation alters mHtt proteostasis, may provide valuable information for the development of new therapies in HD. Using two distinct cellular models of HD, namely, PC12 cells with inducible expression of full-length Htt and U2OS cells transfected with plasmids encoding N-terminal truncated Htt, we studied the proteostasis of both species and how this relates with the chaperone machinery. The molecular chaperone Hsp70 is a main effector of the proteostasis network, acting in client proteins by redirecting them for either folding or degradation, or by promoting its disaggregation. Here, we investigated the effects of the pharmacological modulation of Hsp70 with YM-1, an inhibitor of the Hsp70 ATPase activity on mHtt proteostasis and toxicity. Additionally, since YM-1 may accumulate in mitochondria, we evaluated the effect of YM-1 on mitochondrial function. Hsp70 interacted with full-length mHtt but not wild-type Htt, indicating a specific recognition of the misfolded protein by Hsp70. Neither the expression of full-length or N-terminal mHtt

activated the HSR, thus limiting chaperone availability to deal with mHtt accumulation. The proteostasis of both Htt species was similarly altered by Hsp70 modulation, treatment with YM-1 increased the degradation of both soluble full-length and N-terminal mHtt, possibly via the ubiquitin proteasome system. Hsp70 interaction with N-terminal mHtt aggregates prompted us to investigate YM-1 effect on mHtt aggregation. The absence of a disaggregation complex formation in either control or YM-1 treated cells, observed in N-terminal mHtt expressing cells, can explain the lack of YM-1 effect on insoluble N-terminal mHtt aggregate levels. N-terminal mHtt induced cell death was unaffected by treatment with YM-1, indicating that the decrease in soluble levels of N-terminal mHtt was insufficient to ameliorate mHtt toxicity. Interestingly, results on the levels of the mitochondrial Hsp70, mtHsp70, in both full-length and N-terminal Htt models indicate that YM-1 activates the mitochondrial unfolded protein response, suggesting that YM-1 may affect mitochondrial function. Indeed, preliminary data indicate that YM-1 alters mitochondrial membrane potential. This study supports pharmacological modulation of Hsp70 as a potential strategy to limit mHtt accumulation in HD and provide new insight on potential off-target effects of YM-1 in specific organelles, such as mitochondria.

Keywords: Huntington's disease; Proteostasis; Mitochondria; Molecular chaperones; Hsp70

RESUMO

RESUMO

As doenças de poliglutaminas são um grupo de doenças neurodegenerativas que resultam de expansões de repetições CAG nos genes afetados com a capacidade de codificar tratos de glutaminas expandidos nas proteínas correspondentes que tornam a proteína suscetível de enovelamento aberrante. A proteína com a expansão de poliglutaminas pode mediar a patogénese da doença através de mecanismos que incluem desregulação transcricional, disfunção mitocondrial e proteostasia anormal. A doença de Huntington (DH) é uma doença de poliglutaminas em que a proteína afetada é a huntingtina (Htt). A proteostasia anormal na DH está associada à acumulação e agregação da Htt mutante (mHtt). Pensa-se que a acumulação da mHtt com enovelamento aberrante sobrecarrega a maquinaria de chaperonas, consequentemente desviando um excesso de outras proteínas aberrantes para as vias de degradação e levando a um colapso geral da rede de proteostasia. A bioenergética, dinâmica e o controlo de qualidade mitocondrial também estão alterados na DH. A mHtt pode exercer os seus efeitos na mitocôndria de forma indireta ou por interação direta com o organelo e as suas proteínas associadas. A maquinaria de chaperonas tem um papel essencial na rede de proteostasia, atuando no enovelamento, transporte, desagregação e degradação de proteínas. Em resposta a um insulto proteotóxico, como a acumulação de proteínas aberrantes, a expressão de chaperonas moleculares, conhecidos por proteínas de shock térmico (Hsp), pode ser induzida pela ativação da resposta ao shock térmico (HSR). No entanto, os níveis de diferentes Hsp estão reduzidos em diversos modelos de doenças de poliglutaminas. Os resultados da modelação genética e farmacológica dos componentes da maquinaria de chaperonas moleculares suportam a modelação de Hsp como uma potencial abordagem terapêutica em doenças de poliglutaminas. Contudo, a modelação de Hsp específicos pode exercer diferentes efeitos dependendo da natureza da proteína cliente.

Uma melhor compreensão de como a mHtt interfere com a maquinaria de chaperonas e, por sua vez, como é que a modelação de Hsp altera a proteostasia da mHtt, pode fornecer informação útil para o desenvolvimento de novas terapias na DH. Usando dois modelos celulares de DH distintos, nomeadamente, células PC12 com expressão indutível de Htt completa e células U2OS transfetadas com plasmídeos que codificam Htt truncada N-terminal, estudou-se a proteostasia de ambas as espécies e a sua relação com a maquinaria de chaperonas. A chaperona molecular Hsp70 é um efetor principal da rede de proteostasia, atuando nas proteínas clientes redirecionando-as para enovelamento ou degradação, ou promovendo a sua desagregação. Neste trabalho, investigaram-se os efeitos da modelação farmacológica da Hsp70 com YM-1, um inibidor da atividade ATPase da Hsp70, na proteostasia e toxicidade da mHtt. Adicionalmente, uma vez que o YM-1 se

pode acumular na mitocôndria, avaliou-se o efeito do YM-1 na função mitocondrial. A Hsp70 interagiu com a forma completa da mHtt mas não com a Htt não mutante, indicando um reconhecimento específico da proteína aberrante pela Hsp70. Nem a expressão da mHtt completa nem da N-terminal ativaram a HSR, limitando, assim, a disponibilidade das chaperonas para lidarem com a acumulação da mHtt. A proteostasia de ambas as espécies foi alterada de forma semelhante pela modelação da Hsp70, o tratamento com YM-1 aumentou a degradação tanto da forma completa como da forma N-terminal da mHtt solúvel, possivelmente pela via da ubiqutina-proteossoma. A interação da Hsp70 com os agregados de mHtt N-terminal levou-nos a investigar o efeito do YM-1 na agregação de mHtt. A ausência de formação do complexo de desagregação tanto no controlo como nas células tratadas com YM-1, observada nas células que expressam mHtt N-terminal, pode explicar a falta de efeito do YM-1 nos níveis de agregados insolúveis de mHtt N-terminal. A morte celular induzida pela mHtt N-terminal não foi afetada pelo tratamento com YM-1, indicando que a diminuição dos níveis de mHtt N-terminal solúveis não foi suficiente para aliviar a toxicidade de mHtt.

Interessantemente, os resultados dos níveis da Hsp70 mitocondrial, mtHsp70, em ambos os modelos de Htt completa e Htt N-terminal indicam que o YM-1 ativa a resposta ao desdobramento proteico mitocondrial, sugerindo que o YM-1 pode afetar a função mitocondrial. De facto, dados preliminares indicam que o YM-1 altera o potencial de membrana mitocondrial. Este estudo suporta a modelação farmacológica da Hsp70 como uma potencial estratégia para limitar a acumulação de mHtt na DH e fornece novos dados de potenciais efeitos do YM-1 em organelos específicos como a mitocôndria.

Palavras-chave: Doença de Huntington; Proteostasia; Mitocôndria; Chaperonas moleculares; Hsp70

GENERAL INDEX

GENERAL INDEX

INDEX OF FIGURES

INDEX OF FIGURES

1. Introduction

4. Results

INDEX OF TABLES

INDEX OF TABLES

1. Introduction

LIST OF ABBREVIATIONS

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Abdil	Antibody dilution buffer
AR	Androgen receptor
BAG1	Bcl-2-associated athanogene 1
BSA	Bovine serum albumine
CACNA1A	α1A subunit of the voltage-dependent calcium channel Cav2.1
CHIP	C-terminal Hsc70-interacting protein
DMEM	Dulbecco's modified Eagle medium
DMSO	Dimethyl sulfoxide
DRPLA	Dentatorubral-pallidoluysian atrophy
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
FACS	Fluorescence activated cell sorting
gamitrinibs	Geldanamycin mitochondrial matrix inhibitors
GGA	Geranylgeranylacetone
Grp	Glucose-regulated protein
HD	Huntington's disease
Hip	Hsp70-interacting protein
HS	Heat shock
Hsc70	Constitutive Hsp70
HSF1	Heat shock factor 1
Hsp	Heat shock protein
HSR	Heat shock response
Htt	Huntingtin
LDS	Lithium dodecyl sulfate
mHtt	Mutant Htt
mtHsp70	Mitochondrial Hsp70
mtUPR	Mitochondrial unfolded protein response
NBD	Nucleotide binding domain
NEF	Nucleotide exchange factor
NGF	Nerve growth factor
PBS	Phosphate-buffered saline buffer
PBST	PBS with Tween 20
polyQ	Polyglutamine
RFP	Red Fluorescent Protein
SBD	Substrate-binding domain

SBMA	Spinal and bulbar muscular atrophy
SCA	Spinocerebellar ataxia
SDS	Sodium dodecyl sulfate
STAGA	SPT3/TAF GCN5 complex
TBP	TATA-box binding protein
TFTC	TATA-binding protein-free TAF-containing complex
TIM23	Translocase of the inner membrane
$TMRM^+$	Tetramethylrhodamine methyl ester
TRAP-1	Tumor necrosis factor receptor associated protein-1
UPR	Unfolded protein response
UPS	Ubiquitin proteasome system

THESIS OUTLINE

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This thesis is organized in six main sections:

CHAPTER I – GENERAL INTRODUCTION

This section comprises a general introduction of the main topics addressed in this work, including the pathogenesis of polyglutamine disorders, the constitution and mechanisms of action of the molecular chaperone machinery, as well as the study of the modulation of the chaperone machinery in polyglutamine and other neurodegenerative diseases. The information organized in this section corresponds to the review article developed within the scope of this thesis:

Reis, S. D., B. R. Pinho and J. M. A. Oliveira (2017) Modulation of Molecular Chaperones in Huntington's Disease and Other Polyglutamine Disorders. Mol Neurobiol 54(8): 5829-5854.

CHAPTER II – BACKGROUND AND OBJECTIVES

This section includes a brief 'Huntington's disease and molecular chaperones' focused literature review and a list of open questions in this field to contextualize the thesis research objectives which are addressed in the end of this section.

CHAPTER III – MATERIALS AND METHODS

In this section, all the materials and methodologies used in this thesis are described.

CHAPTER IV – RESULTS

This section presents a detailed description of all the results obtained during the course of this thesis, namely the characterization of the cellular models and the evaluation of the effects of Hsp70 modulation in these models.

CHAPTER V – DISCUSSION AND CONCLUSION

This section discusses the key findings of the experimental work according to the recent available bibliography and ends with a summary of the main conclusions addressing future directions in the field.

CHAPTER VI – REFERENCES

This section lists all the bibliographic references used in the writing of this thesis.

CHAPTER I

GENERAL INTRODUCTION

1. General Introduction

Huntington's disease (HD) belongs to a group of progressive neurodegenerative diseases, named polyglutamine disorders, as it is caused by a mutant form of the huntingtin protein (Htt) with a polyglutamine expansion, which renders the protein highly prone to misfolding and aggregation (Labbadia and Morimoto, 2013; Ross et al., 2014). The Htt interactome comprises proteins involved in diverse cellular processes, such as gene transcription, energy metabolism and proteostasis, which are disrupted upon expression of mutant Htt (mHtt) (Shirasaki et al., 2012; Labbadia and Morimoto, 2013).

Abnormal proteostasis in HD is related to the accumulation and aggregation of mHtt. The current model proposes that the accumulation of misfolded mHtt overwhelms the chaperone machinery, consequently diverting an excess of other misfolded proteins to the degradation pathways and ultimately leading to a global collapse of the proteostasis network (Soares et al., 2019). The pathogenicity of mHtt aggregates is not completely understood. The current hypothesis postulates that mHtt aggregation decreases the levels of toxic diffuse mHtt, removing the trigger for apoptosis, but progressively disrupts cellular homeostasis through sequestration of other essential proteins, such as molecular chaperones (Ramdzan et al., 2017).

Mitochondrial dysfunction in HD results from several anomalies in mitochondrial bioenergetics, dynamics and quality control (Oliveira, 2010b; Guedes-Dias et al., 2016). Mitochondria isolated from cells of HD patients and brains of HD mice models exhibited similar deficits in membrane potential and calcium homeostasis (Panov et al., 2002). Increased mitochondrial fragmentation has been observed in brains of HD patients and mice and associated with an altered expression and activity of proteins involved in fusion and fission (Kim et al., 2010; Shirendeb et al., 2011; Song et al., 2011; Shirendeb et al., 2012). The clearance of defective mitochondria through autophagy (mitophagy) was found to be decreased in HD models, possibly a decreased targeting of ubiquitinated mitochondria to autophagosomes (Khalil et al., 2015). mHtt may exert its effects on mitochondria by direct interaction with the organelle and associated proteins as mHtt was found to interact with mitochondria in different models of HD, being its exact sub-mitochondrial localization still unclear (Oliveira, 2010a; Yano et al., 2014).

Molecular chaperones known by heat shock proteins (Hsp) are key elements in protein misfolding disorders given their role in protein folding, disaggregation and degradation (Reis et al., 2017). In response to a proteotoxic insult, such as the accumulation of misfolded proteins, the expression of Hsp can be induced through activation of the heat shock response (HSR) by heat shock factor 1 (HSF1) (Anckar and Sistonen, 2011; Gomez-Pastor et al., 2018). As for the Hsp isoforms restricted to specific cellular compartments,

such as the endoplasmic reticulum and mitochondrial Hsp, their expression can be induced through activation of the unfolded protein response (UPR) and mitochondrial UPR (mtUPR), respectively. (Lamech and Haynes, 2015; Wang and Kaufman, 2016; Shpilka and Haynes, 2018).

Hsp90 and Hsp70 are the main effectors of the mammalian proteostasis network, acting in a multiprotein complex that includes co-chaperones such as Hsp40 (Kirschke et al., 2014; Taipale et al., 2014; Pratt et al., 2015). Whereas Hsp90 mediates the final folding of client proteins, stabilizing them and inhibiting their degradation, Hsp70 acts in early folding or abnormal folding stages of client proteins, redirecting them for either folding or degradation (Chiosis et al., 2013; Mayer, 2013; Karagoz and Rudiger, 2015). More recently, Hsp70 was also found to play a role in protein disaggregation (Nillegoda et al., 2015; Nillegoda et al., 2018).

The levels of different Hsp are reduced in different models of HD and other polyglutamine disorders, indicating their potential as therapeutic targets in neurodegenerative diseases characterized by the accumulation and aggregation of misfolded proteins (Chou et al., 2008; Yamanaka et al., 2008; Chafekar and Duennwald, 2012; Neueder et al., 2017). Either genetic or pharmacological modulation of molecular chaperones (for molecules structure see Appendix 1 and 2) alters protein levels and aggregation, supporting chaperone modulation as a potential therapeutic approach in polyglutamine disorders. Nevertheless, data on genetic and pharmacological modulation also evidences that the chaperone machinery may exert different effects depending on the nature of the client protein (Reis et al., 2017).

In the following sections the roles and mechanisms of action of members of Hsp90 and Hsp70 families are described, as well as their modulators. A brief description of the 9 diseases that compose the group of the polyglutamine disorders is also included, together with the available data on the genetic and pharmacological modulation of different components of the chaperone machinery. Insights from other neurodegenerative diseases are also considered. This chapter ends addressing future directions in the field.

1.1. Modulation of molecular chaperones in Huntington's disease and other polyglutamine disorders

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Modulation of molecular chaperones in Huntington's disease and other polyglutamine disorders

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Abstract

Polyglutamine expansion mutations in specific proteins underlie the pathogenesis of a group of progressive neurodegenerative disorders, including Huntington's disease, spinal and bulbar muscular atrophy, dentatorubral-pallidoluysian atrophy, and several spinocerebellar ataxias. The different mutant proteins share ubiquitous expression and abnormal proteostasis, with misfolding and aggregation, but nevertheless evoke distinct patterns of neurodegeneration. This highlights the relevance of the full protein context where the polyglutamine expansion occurs and suggests different interactions with the cellular proteostasis machinery. Molecular chaperones are key elements of the proteostasis machinery and therapeutic targets for neurodegeneration. Here we provide a focused review on Hsp90, Hsp70, and their co-chaperones, and how their genetic or pharmacological modulation affects the proteostasis and disease phenotypes in cellular and animal models of polyglutamine disorders. The emerging picture is that, in principle, Hsp70 modulation may be more amenable for long-term treatment by promoting a more selective clearance of mutant proteins than Hsp90 modulation, which may further decrease the necessary wild-type counterparts. It seems, nevertheless, unlikely that a single Hsp70 modulator will benefit all polyglutamine diseases. Indeed, available data, together with insights from effects on tau and alpha-synuclein in models of Alzheimer's and Parkinson's diseases, indicates that Hsp70 modulators may lead to different effects on the proteostasis of different mutant and wild-type client proteins. Future studies should include the further development of isoform selective inhibitors, namely to avoid off-target effects on Hsp in the mitochondria, and their characterization in distinct polyglutamine disease models to account for client protein-specific differences.

Keywords

Heat shock proteins; Hsp70; Huntington's disease; Neurodegeneration; Proteostasis; Mitochondria

1.1.1. Overview

The successful folding and the conformational maintenance of newly synthesized proteins are essential for protein homeostasis (proteostasis). Additionally, cells must have mechanisms to regulate the localization, concentration, and the activity of different proteins in response to intrinsic and extrinsic stimuli (Balch et al., 2008; Hipp et al., 2014). Molecular chaperones are proteins that stabilize or assist the acquisition of the active conformation of other proteins, without being part of their final structure. Molecular chaperones are involved in multiple aspects of proteome maintenance, acting in protein trafficking, folding, aggregation, and degradation (Hartl et al., 2011; Kim et al., 2013). Although molecular chaperones were initially described as heat shock proteins (Hsp; as the first members were discovered to be upregulated under such stress conditions; (Lindquist, 1986)), it is currently known that most molecular chaperones are constitutively expressed and involved in maintaining proteostasis at any time (Karagoz and Rudiger, 2015).

Molecular chaperone families (Hsp40, Hsp60, Hsp70, Hsp90, Hsp100, and the small Hsp) were named according to the molecular weight of their members. Hsp100 has an important role in protein disaggregation in non-metazoans (e.g. yeast), but metazoans lack Hsp100 in the cytosol and nucleus (Kirstein et al., 2009; Doyle et al., 2013). Hsp60 chaperones are present in the cytosol and the mitochondria of eukaryotes, but seem to interact with a higher percentage of proteins in bacteria and archaea (Hartl et al., 2011; Smith et al., 2015). The small Hsp are ubiquitous chaperones that bind to non-native proteins, preventing their aggregation and facilitating further refolding or degradation (Eyles and Gierasch, 2010; Basha et al., 2012; Kampinga and Garrido, 2012). Together, molecular chaperones are estimated to account for approximately 10% of the cellular mass, with Hsp90 and Hsp70 alone representing half of that (Shrestha et al., 2016). Indeed, Hsp90 and Hsp70 are the main effectors of the mammalian protein homeostasis network, acting in a multiprotein complex that includes co-chaperones such as Hsp40 (Kirschke et al., 2014; Taipale et al., 2014; Pratt et al., 2015). Hsp90 typically interacts with client proteins in their late stages of folding. Hsp90 stabilizes and mediates the final folding of client proteins, preserving their activity and inhibiting their degradation (Chiosis et al., 2013; Karagoz and Rudiger, 2015). In contrast, Hsp70 typically interacts with proteins at an early stage of folding or with proteins with an abnormal folding, but not with their folded counterparts. The binding of Hsp70 to unfolded or misfolded proteins allows redirecting client proteins for either folding or degradation (Mayer, 2013; Karagoz and Rudiger, 2015). Hsp70 is also relevant in the context of protein aggregation, being able to bind protein aggregates and promote their disaggregation (Gao et al., 2015; Nillegoda et al., 2015).

Defects in proteostasis are implicated in the process of aging and in the pathogenesis of several degenerative conditions, including the polyglutamine (polyQ) expansion disorders (Kaushik and Cuervo, 2015; Labbadia and Morimoto, 2015). PolyQ disorders comprise Huntington's disease (HD), spinal and bulbar muscular atrophy (SBMA), six of the spinocerebellar ataxias (SCA 1-3, 6, 7, 17), and dentatorubral-pallidoluysian atrophy (DRPLA), and all stem from expanded CAG repeats in the coding regions of the affected genes, resulting in mutant proteins with expanded glutamine tracts (Blum et al., 2013). These disorders further share an inverse correlation between the CAG repeat number and the age of onset, and are primarily neurodegenerative despite ubiquitous expression of the respective mutant protein that forms insoluble aggregates in affected neurons (Orr and Zoghbi, 2007; Katsuno et al., 2012; Orr, 2012a). Additionally, nuclear localization of the expanded protein seems to be required for the induced toxicity in the majority of polyQ disorders (Orr and Zoghbi, 2007; Orr, 2012a). Interestingly, although all these diseases share polyQ expansion in the affected protein, they affect different regions of the neural tissue (Blum et al., 2013). This differential neurodegeneration highlights the relevance of the protein context where the polyQ is inserted, and strongly suggests that the different mutant polyQ proteins and their wild-type forms may interact differently with components of the molecular chaperone machinery, justifying a focused review.

The modulation of molecular chaperones has been mainly studied in the context of cancer therapy (Hong et al., 2013; Sherman and Gabai, 2015). Evidence suggests, however, that molecular chaperones are also potential therapeutic targets for neurodegenerative diseases characterized by the accumulation and aggregation of misfolded proteins. Indeed, molecular chaperones co-localize with polyQ-containing insoluble aggregates and their expression is decreased in several polyQ disease models, suggesting that their modulation is worth investigating as a potential therapeutic approach (Hay et al., 2004; Chou et al., 2008; Yamanaka et al., 2008; Gao et al., 2011; Chafekar and Duennwald, 2012). This review focuses on the modulation of molecular chaperones in the context of polyQ disorders. The first section describes the roles and mechanisms of action of key members of the Hsp90 and Hsp70 families, together with their standard and novel small-molecule modulators. The second section integrates the available data on the genetic and pharmacological modulation of Hsp90/70 and associated chaperones in models of HD and other polyQ disorders, addressing how treatments affect mutant protein levels, aggregation, survival and other key disease phenotypes. This review ends with insights from other main neurodegenerative diseases, and a discussion including future directions in the field.

1.1.2. Molecular chaperones and the heat shock response

The heat shock response is a highly conserved cellular reaction to proteotoxic insults such as heat, oxidative stress and toxins. In mammals, the master regulator of this response is the constitutively expressed heat shock factor 1 (HSF1), which acts by inducing the transcription of Hsp genes. Proteotoxic insults activate HSF1, converting its inactive monomers into trimers with DNA binding activity and transactivation capacity (Akerfelt et al., 2010; Anckar and Sistonen, 2011). Interaction with Hsp90 prevents the activation of monomeric or trimeric HSF1. Similarly, interaction with Hsp70 and Hsp40 inhibits HSF1 transactivation capacity (Figure 1.1.1). These interactions seem to act as regulatory feedback mechanisms that coordinate HSF1 activity with the expression of its HSP targets, and with the state of the protein-folding environment (Akerfelt et al., 2010; Anckar and Sistonen, 2011; Neef et al., 2011).

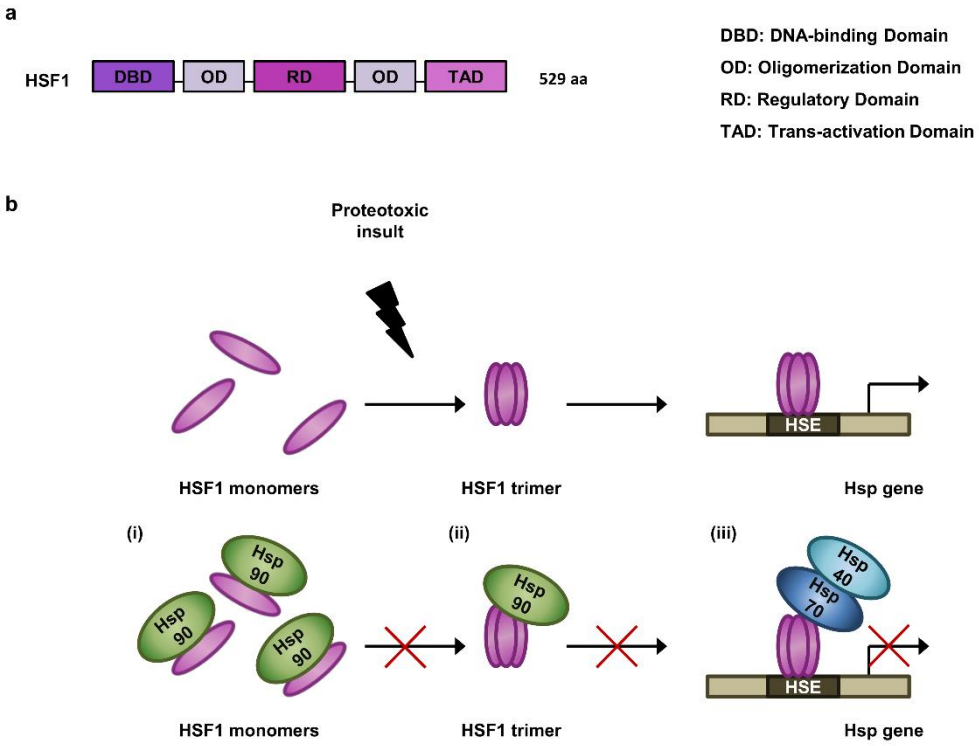

Figure 1.1.1 Regulation of Hsp transcription by heat shock factor 1 (HSF1). a Domain structure of human HSF1 (NCBI accession number: NP_005517.1). **b** Proteotoxic insults convert inactive monomers of HSF1 into trimers with DNA binding activity. HSF1 trimers bind heat shock elements (HSE) and induce transcription of HSP genes. The availability of heat shock proteins exerts a feedback regulation on HSF1 activity: Hsp90 can interact with either (i) monomeric or (ii) trimeric HSF1, preventing their activation; (iii) Hsp70 and Hsp40 can inhibit the transactivation capacity of HSF1 (Akerfelt et al., 2010; Anckar and Sistonen, 2011; Neef et al., 2011).

1.1.2.1. The Hsp90 family

Hsp90 is a highly dynamic family of proteins, capable of adopting several distinct conformations, and being regulated by multiple interactors (Saibil, 2013). This chaperone family has multiple client proteins, including steroid receptors, kinases, and several other unrelated proteins, including intrinsically disordered proteins such as tau (Mayer et al., 2009; Karagoz et al., 2014; Kirschke et al., 2014; Lorenz et al., 2014; Verba et al., 2016). Clients are delivered to Hsp90 by Hsp70 and other co-factors (Kim et al., 2013). Hsp90 typically interacts with client proteins in their late stages of folding by binding to hydrophobic residues scattered over a large surface area (Mayer, 2013; Karagoz and Rudiger, 2015). Hsp90 may stabilize these proteins, mediate their folding and bring about their activation, but Hsp90 is also involved in directing clients to proteasomal degradation (Chiosis et al., 2013).

The Hsp90 family belongs to the gyrase, histidine kinase, and MutL superfamily of ATPases (Kim et al., 2013). Hsp90 is constituted by three domains: an N-terminal nucleotide binding domain (NBD) followed by a charged linker region, a middle domain – involved in client protein binding (although other domains may participate in client binding (Rohl et al., 2013; Karagoz et al., 2014)), and a C-terminal domain (Mayer et al., 2009) (Figure 1.1.2a). Importantly, Hsp90 is active in the form of a dimer, assembled via interaction sites located in the C-terminal domain. This domain also mediates Hsp90 interactions with several co-chaperones (Kim et al., 2013).

The Hsp90 dimer undergoes an ATP-regulated cycle, similarly to other chaperones. Client protein binding affinity is regulated by the combined effects of ATP binding and hydrolysis, post-translational modifications, and interactions with co-chaperones (Kim et al., 2013; Saibil, 2013; Karagoz and Rudiger, 2015) (Fig. 2b). Client proteins themselves also influence the conformational equilibrium in interplay with co-chaperones, thus acting as modulators of the Hsp90 machinery (Karagoz et al., 2014; Lorenz et al., 2014).

In eukaryotes, Hsp90 can be found in the cytosol, nucleus and organelles such as mitochondria and the endoplasmic reticulum. The nuclear localized Hsp90 represents only a small fraction of the cytosolic Hsp90, which translocates to the nucleus in response to stress and other stimuli (Taipale et al., 2010; Li et al., 2012). In humans, Hsp90 exists as four isoforms: Hsp90α and Hsp90β represent the cytosolic forms, Grp (glucose-regulated protein) 94 is found in the endoplasmic reticulum (ER), and TRAP-1 (tumor necrosis factor receptor associated protein-1) is associated with mitochondria (Johnson, 2012) (Table 1.1.1).

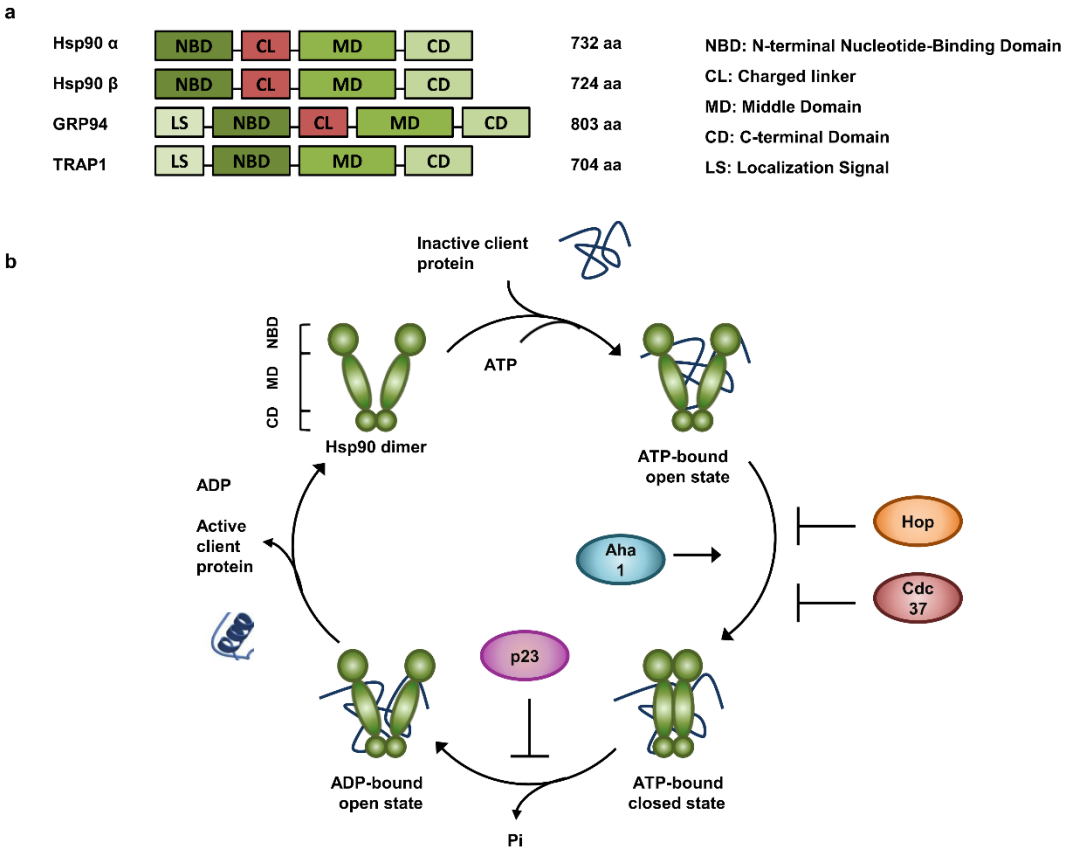

Figure 1.1.2. The Hsp90 reaction cycle. a Domain structure of human Hsp90 family members (NCBI accession numbers: Hsp90α, AAI21063.1; Hsp90β, AAH68474.1; GRP94, AAH66656.1; TRAP-1, AAH23585.1). **b** The ATP-bound open state of the Hsp90 dimer interacts with inactive client proteins transferred from the Hsp70 system by the co-chaperone Hop (Hsp70-Hsp90 organizing protein). Hop and co-chaperones such as Cdc37 (cell division cycle 37 homolog) stabilize the open conformation of Hsp90, facilitating client binding and inhibiting ATP hydrolysis, whereas the activator of Hsp90 ATPase, Aha1, induces the transition to the closed conformation. The co-chaperone p23 stabilizes the Hsp90 closed conformation, trapping the client protein. After ATP hydrolysis, the client protein is released in an active state (Kim et al., 2013; Rohl et al., 2013; Mayer and Le Breton, 2015).

1.1.2.1.1. Grp94 – the endoplasmic reticulum Hsp90 isoform

Grp94 is found in the endoplasmic reticulum, and is the only Grp in the Hsp90 chaperone family (Table 1.1.1) (Marzec et al., 2012; Lee, 2014) . In addition to Grp94, there are other Grp present in the endoplasmic reticulum and also in the mitochondria. Collectively, Grp

are chaperones induced upon ER stress, a state usually associated with ER calcium depletion and/or accumulation of misfolded proteins in the ER (Lee, 2014). At least one third of the proteins in eukaryotic cells are synthesized at the ER membrane. After their synthesis, these proteins are translocated into the ER lumen where they acquire their functional structure. When proteins are properly folded, they undergo vesicular-mediated transport through the organelles of the secretory pathway (Behnke et al., 2015; Zhu and Lee, 2015; Wang and Kaufman, 2016). When protein misfolding prevails, the unfolded protein response is activated, which is a mechanism that promotes ER protein-folding homeostasis. The unfolded protein response involves a transient reduction of protein synthesis, while enhancing protein folding, transport and ER-associated protein degradation and autophagy (Melnyk et al., 2015; Zhu and Lee, 2015; Wang and Kaufman, 2016).

Unlike cytosolic Hsp90, Grp94 has no known co-chaperones (Marzec et al., 2012). Grp94 contributes to the ER protein quality control and calcium storage, being one of the major calcium binding proteins (Eletto et al., 2010). Free calcium levels regulate Grp94 chaperone activity via a calcium binding site, whose occupation enhances Grp94 association with client proteins (Biswas et al., 2007). The Grp94 molecular chaperone activity includes the direct folding and/or assembly of secreted and membrane proteins, and assists the targeting of misfolded proteins for degradation (Eletto et al., 2010; Marzec et al., 2012).

1.1.2.1.2. TRAP-1 – the mitochondrial Hsp90 isoform

TRAP-1 is a Hsp90 isoform that is predominantly found in the mitochondrial matrix, with a smaller fraction in the intermembrane space (Table 1.1.1) (Altieri, 2013). Like Grp94, TRAP-1 has no known co-chaperones (Altieri, 2013). TRAP-1 functions are incompletely understood but do not seem to overlap with the functions of other Hsp90 chaperones, and might be crucial for mitochondrial physiology (Kang et al., 2007; Rasola et al., 2014). The most extensively reported TRAP-1 function is cytoprotection, via an interaction with cyclophilin D that prevents mitochondrial permeability transition pore opening (Kang et al., 2007), and via decreasing reactive oxygen species by yet unknown mechanisms (Rasola and Bernardi, 2015). Additionally, TRAP-1 has been associated with mitochondrial morphology, through regulation of fission proteins (Takamura et al., 2012); with mitophagy, through improved molecular quality control that reduces the number of damaged mitochondria (Costa et al., 2013); with the maintenance of a functional electron transport chain, by upregulation of complex I activity (Costa et al., 2013; Zhang et al., 2013); and with the ER stress response (Takemoto et al., 2011).

TRAP-1 was also found on the outer side of the ER and shown to regulate the ubiquitination of specific proteins destined to mitochondria through interaction with TBP7 (a component of the 19S proteasome subunit). Data support the hypothesis that TRAP-1 and TBP7 perform the quality control of proteins destined to mitochondria at the ER-mitochondria interface: TRAP-1 avoids the mitochondrial import of damaged proteins, sequestering them to be refolded (Amoroso et al., 2012).

Table 1.1.1. Proteins of the human Hsp90 family.

Gene	Protein designations [a]	Localization	Stress inducible	Identity (%) [b]
HSP90AA1	Heat shock protein 90kDa alpha class A member 1; **Hsp90α**; Hsp86; Hspc1	Cytoplasm, Nucleus	Yes	100
HSP90AB1	Heat shock protein 90kDa alpha class B member 1; **Hsp90β**; Hsp84; Hspc3	Cytoplasm, Nucleus	No	86
HSP90B1	Heat shock protein 90kDa beta member 1; **Grp94**; Gp96; endoplasmin; Hspc4	Endoplasmic reticulum	Yes	46
TRAP1	TNF receptor-associated protein 1; **TRAP1**; Hsp75; Hspc5	Mitochondria	-	30

[a] Protein designations used in this review are in **bold**.

[b] BLASTP results relative to the HSP90AA1 sequence on the NCBI database. Accession numbers: AAI21063.1; AAH68474.1; AAH66656.1; AAH23585.1.

1.1.2.1.3. Hsp90 modulators

Hsp90 inhibitors are thought to decrease levels of client proteins by preventing their cycling with Hsp90, thereby diverting them to the ubiquitin proteasome system (UPS) for degradation (Garcia-Carbonero et al., 2013). Non-selective Hsp90 inhibitors include naturally occurring compounds, such as geldanamycin and radicicol. Attempts to overcome pharmacological and toxicity issues sprouted derivatives of these drugs, and also purine analogues (e.g. BIIB021 and PU-H71) as improved Hsp90 inhibitors. Geldanamycin derivates include tanespimycin (17-AAG), alvespimycin (17-DMAG), retaspimycin (IPI-504), and the primary active metabolite of tanespimycin (IPI-493/17-AG). Radicicol derivatives include ganetespib (STA-9090), AT-13387, KW-2478, NVP-AUY922, and the orally available NVP-HSP990. All the aforementioned drugs inhibit Hsp90 by competing with nucleotides for their binding site at the N-terminal, shifting the balance to client protein dissociation and thereby allowing its degradation (Wang, 2011; Garcia-Carbonero et al., 2013; Bhat et al., 2014). Interestingly, it has been recently proposed that Hsp90 inhibitors that block the ATP binding pocket may impact on Hsp70 functions, given that the ATP cycles of Hsp90 and Hsp70 may be tightly coupled (Kirschke et al., 2014). Other strategies being pursued to inhibit Hsp90 include the targeting of the Hsp90 C-terminal domain in an attempt

to inhibit binding with co-chaperones (Wang, 2011; Garcia-Carbonero et al., 2013; Bhat et al., 2014). In the context of neurodegenerative disorders, radicicol, geldanamycin, and the geldanamycin derivatives 17-AAG and 17-DMAG are the most frequently tested molecules, as addressed in section 3.

Strategies to identify isoform selective Hsp90 inhibitors are beginning to emerge. Most compounds are being targeted to regions outside the highly conserved nucleotide-binding site (Bhat et al., 2014), but minor differences in the ATP-binding pockets are also being explored to develop Grp94 selective compounds (Duerfeldt et al., 2012). Small-molecule Hsp90 inhibitors that selectively accumulate in mitochondria were also synthesized to preferentially target TRAP-1. In these compounds, designated as geldanamycin mitochondrial matrix inhibitors (gamitrinibs), the ATPase inhibitory component of 17-AAG is fused to a mitochondrial targeting moiety (triphenylphosphonium cation or 1 to 4 cyclic guanidinium repeats). Gamitrinibs were found to accumulate in mitochondria and inhibit the ATPase activity of TRAP-1 with in vitro and in vivo efficacy (Kang et al., 2009).

1.1.2.2. The Hsp70 family

The Hsp70 chaperone family is a ubiquitous class of proteins involved in several steps of the protein quality control, including protein folding, transport and degradation (Saibil, 2013). Hsp70 recognizes short and highly hydrophobic amino acid sequences, which often are integral components of the hydrophobic core of the proteins. Consequently, Hsp70 acts in unfolded and misfolded proteins that have these amino acids exposed and not in their native counterparts (Mayer, 2013).

Hsp70 contain an NBD and a C-terminal substrate-binding domain (SBD) connected by a conserved hydrophobic linker region (Figure 1.1.3a). Hsp70 activity depends on its dynamic interaction with Hsp40 co-chaperones (also called J proteins), the Hsp70-interacting protein (Hip), and nucleotide exchange factors (NEFs), which together regulate the nucleotide state of the Hsp70 ATPase domain. ATP binding or hydrolysis at the NBD alter the conformation of the SBD and, consequently, regulate Hsp70 interaction with client proteins. ATP binding opens the substrate-binding site, allowing interaction with client proteins. In contrast, ATP hydrolysis, which is promoted by Hsp40, closes the substrate-binding site, stabilizing the client protein binding (Kampinga and Craig, 2010; Saibil, 2013). Hip and NEFs interact with the NBD, in a mutually exclusive manner, to regulate client release: an Hip dimer locks ADP in the binding cleft, delaying client release (Li et al., 2013), whereas a NEF releases ADP and, consequently, the client protein

(Kampinga and Craig, 2010; Saibil, 2013). Proteins unable to fold properly may rebind to Hsp70 or be redirected to other chaperones or degradation pathways (Figure 1.1.3b). In addition to its role in protein folding and degradation, Hsp70 is also involved in protein disaggregation, acting in cooperation with Hsp40 co-chaperones and the Hsp110 NEF in the formation of a chaperone complex with disaggregase activity (Nillegoda and Bukau, 2015; Nillegoda et al., 2015) (Figure 1.1.3c).

There are at least 14 Hsp70 isoforms in humans encoded by different genes (Table 1.1.2). Although the vast majority of Hsp70 proteins are mainly cytosolic and nuclear in localization, there are also Hsp70 members restricted to specific compartments, such as mitochondria and the ER. The four most studied Hsp70 isoforms are: the stress induced Hsp70 (Hsp72); the constitutive Hsp70 (Hsc70); the Grp78 or BiP, which is mainly located in the ER; and the mitochondrial Hsp70 (mtHsp70) (Lu et al., 2010).

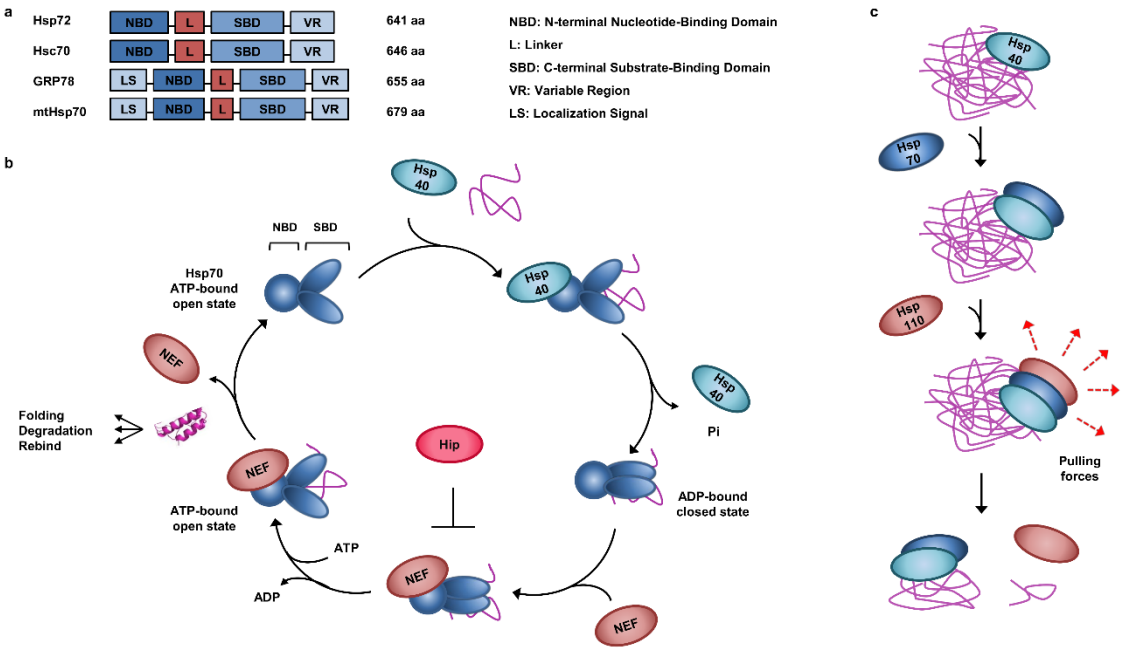

Figure 1.1.3. Hsp70 reaction cycle. a Domain structure of human Hsp70 family members (NCBI accession numbers: Hsp72, AAH18740.1; Hsc70, AAH19816.1; GRP78, AAI12964.1; mtHsp70, AAH24034.1). **b** ATP binding promotes the opening of the substrate-binding site, allowing interaction of Hsp70 with unfolded proteins recruited by the co-chaperone Hsp40. Hsp40 stimulates ATP hydrolysis, inducing the shutting of the substrate-binding site and, consequently, the transition to the ADP-bound closed conformation. Hsp70-interacting protein (Hip) locks ADP in the nucleotide binding domain, delaying substrate release. In contrast, nucleotide exchange factors (NEFs), which have a high affinity for Hsp70 when bound to ADP, trigger ADP dissociation and induce protein release

upon rebinding of ATP. After dissociation from Hsp70, proteins incapable of proper folding may rebind, or be transferred to downstream chaperones or to the degradation machinery (Kampinga and Craig, 2010; Kim et al., 2013; Saibil, 2013). **c** Simplified model of Hsp70-dependent protein disaggregation. Hsp40 co-chaperones (J proteins) target protein aggregates and recruit Hsp70. Hsp110 is also recruited to form a chaperone complex with Hsp70 and Hsp40, whose cooperative pulling forces (dashed red arrows) promote protein disaggregation. The NEF activity of Hsp110 triggers peptide release from the aggregate (Nillegoda and Bukau, 2015; Nillegoda et al., 2015).

1.1.2.2.1. The constitutive Hsc70 and the inducible Hsp72

The major constitutively active form of Hsp70 is Hsc70. Hsc70 is by far the most expressed Hsp70 in all tissues and is only mildly induced during stress conditions. Hsc70 and the inducible Hsp72 have high identity (87%; Table 1.1.2), and share major functions in protein folding, translocation, degradation and prevention of aggregation (Liu et al., 2012). Still, differences in their C-terminal domain may account for some functional differences (Ahn et al., 2005). Indeed, while Hsp72 knockout mice survive, Hsc70 knockout is lethal, thus suggesting different survival roles for these isoforms (Daugaard et al., 2007). Moreover, while under normal conditions Hsc70 regulates several components of the synapse (e.g. N-type calcium channels, neurotransmitter synthesis and packaging, and vesicle recycling), Hsp72 only seems to function at the synapse under stress conditions (Lu et al., 2010). Furthermore, Hsc70 is the only chaperone with a crucial role in recognizing substrates for chaperone-mediated autophagy (Kaushik and Cuervo, 2012).

1.1.2.2.2. Grp78 – the endoplasmic reticulum Hsp70 isoform

The most abundant ER chaperone is the Grp78, which shares 64% identity with Hsp72 (Maattanen et al., 2010) (Table 1.1.2). Similarly to the ER located Hsp90 isoform (Grp94), Grp78 acts as a calcium binding protein, functions in protein folding, and targets proteins for secretion or for the ER-associated degradation. The mechanisms involved in these processes are similar between Grp78 and the previously described Grp94 (Behnke et al., 2015; Zhu and Lee, 2015).

1.1.2.2.3. mtHsp70 – the mitochondrial Hsp70 isoform

mtHsp70 is constitutively expressed and has 52% sequence identity with Hsp72 (Table 1.1.2). It resides mainly in the mitochondria where it acts as the ATP-hydrolyzing subunit of TIM23 (translocase of the inner membrane; (Kang et al., 1990)), assisting the import and folding of nucleus-encoded mitochondrial precursor proteins (Chacinska et al., 2009; Stricher et al., 2013). Such precursor proteins contain targeting signals recognizable by receptors at the mitochondrial surface that target the precursors to different mitochondrial subcompartments (Schmidt et al., 2010). Mitochondrial membrane potential per se is not enough to drive the complete translocation of proteins into the matrix. The additional energy required for this translocation via TIM23 comes from mtHsp70-dependent ATP hydrolysis (Dudek et al., 2013). mtHsp70 binds the unfolded polypeptide chain and drives its movement into the matrix (Schmidt et al., 2010), acting in the subsequent protein folding (van der Laan et al., 2010).

Table 1.1.2. Proteins of the human Hsp70 family.

Gene	Protein designations [a]	Localization	Stress inducible	Identity (%) [b]
HSPA1A/B	Heat shock 70kDa protein 1A/B; Hsp70-1/-2; **Hsp72**	Cytoplasm, Nucleus	Yes	100
HSPA1L	Heat shock 70kDa protein 1-like; Hsp70-1l; Hsp70-HOM	Cytoplasm, Nucleus	No	90
HSPA2	Heat shock 70kDa protein 2	Cytoplasm, Nucleus	No	85
HSPA4	Heat shock 70kDa protein 4; Hsp70RY	Cytoplasm	Yes	33
HSPA4L	Heat shock 70kDa protein 4L; APG-1; Osp94	Cytoplasm	Yes	33
HSPA5	Heat shock 70kDa protein 5; **Grp78**; BiP	Endoplasmic reticulum	Yes	64
HSPA6	Heat shock 70kDa protein 6; HSP70B'	Cytoplasm, Nucleus	Yes	83
HSPA7	Heat shock 70kDa protein 7; HSP70B	Cytoplasm, Nucleus	Yes	84
HSPA8	Heat shock 70kDa protein 8; **Hsc70**; Hsp73	Cytoplasm, Nucleus	No	87
HSPA9	**mtHsp70**; Heat shock 70kDa protein 9; Grp75	Mitochondria	No	52
HSPA12A	Heat shock 70kDa protein 12A	Cytoplasm, Nucleus	-	26
HSPA12B	Heat shock 70kDa protein 12B	Cytoplasm, Nucleus	-	25
HSPA13	Heat shock 70kDa protein 13; STCH	Endoplasmic reticulum	No	39
HSPA14	Heat shock 70kDa protein 14; Hsp70L1	Cytoplasm	Yes	35

[a] Protein designations used in this review are in **bold**.

[b] BLASTP results relative to the HSPA1A sequence on the NCBI database. Accession numbers: AAH18740.1; NP_005518.3; AAH36107.1; NP_002145.3; NP_055093.2; AAI12964.1; NP_002146.2; P48741.2; AAH19816.1; AAH24034.1; NP_079291.2; AAI43933.1; P48723.1; Q0VDF9.1.
Adapted from (Lu et al., 2010; Stricher et al., 2013).

1.1.2.2.4. Hsp70 modulators

Different compounds have been identified and developed to target different aspects of Hsp70 function, but thus far all lack selectivity for individual Hsp70 isoforms. The first was 15-deoxyspergualin, which targets the Hsp70 SBD and allosterically stimulates Hsp70 ATPase activity (Nadeau et al., 1994; Brodsky, 1999). Other compounds that interact with the Hsp70 SBD are 2-phenylethynesulfonamide, also known as pifithrin-μ (Leu et al., 2009), and geranylgeranylacetone (GGA) (Otaka et al., 2007). Pifithrin-μ inhibits Hsp70 folding activity by disrupting its association with co-chaperones, such as Hsp40, and with client proteins (Leu et al., 2009). The mechanism of action of GGA is still incompletely understood; being suggested that GGA binding to Hsp70 promotes its dissociation from HSF1, allowing HSF1 activation (Otaka et al., 2007).

The majority of the developed Hsp70 modulators target the NBD, either stimulating or inhibiting Hsp70 ATPase activity. Those that stimulate Hsp70 ATPase activity include the dihydropyrimidines SW02 and 115-7c (Jinwal et al., 2009; Wisen et al., 2010), whereas Hsp70 ATPase inhibitors include the dihydropyridine CE12 (Chafekar et al., 2012), VER155008 (Massey et al., 2010), myricetin (Jinwal et al., 2009; Chang et al., 2011), and apoptozole (Ko et al., 2015). Compounds such as methylene blue and azure C were also reported to inhibit Hsp70 ATPase activity (Jinwal et al., 2009), possibly by oxidizing residues at the NBD (Miyata et al., 2012).

The rhodocyanine MKT-077 targets the NBD inhibiting Hsp70 ATPase activity and stabilizing the ADP-bound conformation (Rousaki et al., 2011). Researchers have been working to create MKT-077 analogs with reduced toxicity and increased potency and permeability. Improved MKT-077 analogs include YM-01 and the blood brain barrier permeable neutral analog YM-08 (Koren et al., 2012; Miyata et al., 2013). These compounds mimic the ADP-locking activity of the co-chaperone Hip at the NBD, increasing Hsp70 affinity for clients and consequently preventing their aggregation and facilitating transfer to downstream chaperones or degradation machineries (Li et al., 2013; Wang et al., 2013).

1.1.2.3. The chaperone machinery and protein quality control

The Hsp70 and Hsp90 chaperone families play a major role in the proteostasis network, acting together with their co-chaperones as a multiprotein complex in protein quality control (Hartl et al., 2011; Taipale et al., 2014; Pratt et al., 2015). Hsp90 stabilizes the clients, inhibiting their ubiquitination and degradation, whereas Hsp70 promotes the ubiquitination and degradation of proteins that cannot be properly folded. Indeed, when Hsp90 cannot cycle with the client protein, ensuring the native folding state of the client protein, Hsp70 can redirect the misfolded protein to degradation pathways, such as chaperone-assisted proteasomal degradation or autophagy-associated pathways (Kettern et al., 2010; Pratt et al., 2014). The latter pathways include chaperone-assisted selective autophagy and chaperone-mediated autophagy; both are associated with Hsc70 and may compensate for defects in the UPS, which is otherwise the main system responsible for the degradation of misfolded proteins (Koga et al., 2011; Minoia et al., 2014).

The UPS relies on the coupling of ubiquitin chains to target proteins, signaling them for proteasomal degradation (Dantuma and Bott, 2014). After protein recognition by chaperones, chaperone-dependent E3 ubiquitin ligases target E2 ubiquitin-conjugating enzymes to the protein, promoting its ubiquitination (Pratt et al., 2015). The co-chaperone CHIP is an E3 ligase that binds to both Hsp70 and Hsp90, and interacts with E2 ubiquitin-conjugating enzymes through a C-terminal U-box domain, thereby connecting chaperone complexes to the ubiquitination machinery (Edkins, 2015). The co-chaperone BAG1 (bcl-2-associated athanogene 1) associates with Hsp70 and CHIP, promoting protein ubiquitination (Kim et al., 2013) and linking the chaperone system to the proteasome (Gamerdinger et al., 2009; Kettern et al., 2010) (Figure 1.1.4). Interestingly, although Hsp90 is considered to be primarily involved in client protein stabilization and inhibition of degradation, evidence suggests that Hsp90 may also promote the degradation of specific client proteins such as tau, a protein known to misfold and accumulate in Alzheimer's disease (Dickey et al., 2007; Thompson et al., 2012).

In the context of neurodegenerative disorders, the chaperone-assisted proteasomal degradation model presents two possible approaches to promote the degradation of aggregation-prone client proteins. The first is the inhibition of client protein stabilization by Hsp90, inhibiting the cycling of the not-yet-unfolded proteins with Hsp90 to promote its degradation. However, Hsp90 interacts with hundreds of proteins, therefore long-term treatment with Hsp90 inhibitors may lead to the unspecific degradation of multiple Hsp90 client proteins. Thus, the second possible pathway seems to be a better approach, which is the promotion of Hsp70-dependent degradation of already-unfolded or misfolded client proteins (Pratt et al., 2015).

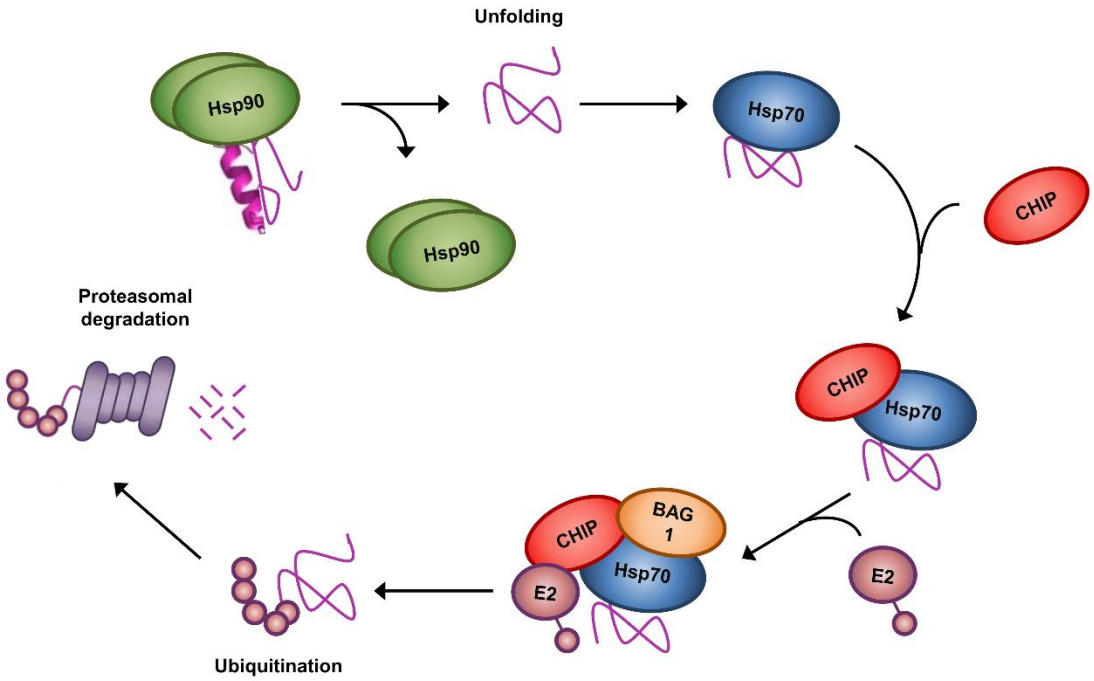

Figure 1.1.4. Simplified model of Hsp90/Hsp70 chaperone machinery in client protein degradation. Dissociation from the Hsp90 machinery allows the unfolding of the client protein. Hsp70 binds the unfolded protein and recruits the chaperone-dependent E3 ubiquitin ligase CHIP. CHIP then targets E2 ubiquitin-conjugating enzymes to the unfolded protein promoting its ubiquitination and subsequent proteasomal degradation. This simplified model (adapted from (Wang et al., 2013)) does not include all possible pathways, being noteworthy that Hsp90 and CHIP may form a complex that is proposed to recognize and target for degradation clients such as phosphorylated tau (Dickey et al., 2007).

1.1.3. Modulation of molecular chaperones in Huntington's disease and other polyglutamine disorders

The expanded polyQ tract renders the affected protein highly prone to aggregation. Still, among polyQ disorders, aggregates may present different subcellular localizations, and different neuronal populations show the highest vulnerability (Gatchel and Zoghbi, 2005; Blum et al., 2013; Rub et al., 2013). Also, while the aggregates clearly indicate that proteostasis is altered, it remains uncertain if aggregates are the main mediators of neuronal dysfunction (Walsh and Selkoe, 2016). Indeed, aggregate formation may actually reduce the diffuse levels of the mutant protein and the associated risk of neuronal death (Arrasate et al., 2004). Nevertheless, protein aggregates do sequester several proteins,

including components of the transcriptional and protein quality control machineries, thus suggesting a role in transcriptional dysregulation and abnormal proteostasis that are common features to all polyQ disorders (Gatchel and Zoghbi, 2005; Labbadia and Morimoto, 2013). Either genetic or pharmacological modulation of molecular chaperones alters protein levels and aggregation, thus being potential therapeutic approaches for polyQ disorders (Gao et al., 2011; Chafekar et al., 2012; Wang et al., 2013).

1.1.3.1. Huntington's disease

In HD, the polyQ expansion occurs in the huntingtin (Htt) protein (Table 1.1.3). Htt interacts with multiple proteins involved in diverse cellular processes (e.g. gene transcription, energy metabolism, cell signaling, and proteostasis), which are disrupted upon expression of mutant Htt; mHtt) (Shirasaki et al., 2012; Labbadia and Morimoto, 2013). Key components of HD pathology are mitochondrial dysfunction, transcriptional dysregulation and abnormal proteostasis (Schapira et al., 2014).

Mitochondrial bioenergetics and dynamics are disturbed by expression of mHtt (Oliveira, 2010b; Oliveira and Lightowlers, 2010; Guedes-Dias et al., 2016). mHtt was shown to interact with mitochondria in both cellular and animal models, however, its exact sub-mitochondrial localization is still unclear (Oliveira, 2010a; Yano et al., 2014). mHtt was reported to disrupt mitochondrial protein import via direct interaction with the TIM23 complex (Yano et al., 2014), whose ATP-hydrolysing subunit is the chaperone mtHsp70 (Kang et al., 1990; Dudek et al., 2013).

Transcriptional dysregulation in HD results from mHtt interaction with major components of the transcriptional machinery. In the context of chaperones, mHtt was found to interact with and sequester the transcription factor NF-Y in brains of HD mice. As this sequestration reduces the NF-Y dependent Hsp70 transcription, it may explain the reduced expression of Hsp70 in HD, and contribute for abnormal proteostasis (Yamanaka et al., 2008).

Abnormal proteostasis in HD relates to the aggregation-prone features of mHtt, which seed nuclear and cytoplasmic aggregates that sequester transcription factors and quality control proteins, thereby disrupting normal transcription and protein clearance (Labbadia and Morimoto, 2013). Indeed, the two major protein clearance pathways, UPS and autophagy, seem compromised in HD. Ubiquitin chains do accumulate in HD brains (Bennett et al., 2007), however, this is not necessarily due to direct proteasome inhibition by mHtt. Instead, mHtt may overwhelm the proteostasis network, leading to increased levels of other misfolded proteins that are diverted to the UPS where they compete for the limited

degradation capacity of the 26S proteasome (Hipp et al., 2012). Concerning autophagy, data suggest transcriptional dysregulation of autophagy-related genes in HD brains (Hodges et al., 2006). Also, mHtt may impair autophagosome trafficking (Wong and Holzbaur, 2014) and cargo recognition (Hodges et al., 2006; Martinez-Vicente et al., 2010; Wong and Holzbaur, 2014; Guedes-Dias et al., 2016). Still, the hypothesis that wild-type Htt may function as a scaffold for selective autophagy cautions about the risk of decreasing both wild-type and mHtt with non-selective therapeutic strategies (Ochaba et al., 2014).

1.1.3.1.1. Genetic modulation of molecular chaperones in HD

Proteomic analysis of the mHtt interactome revealed that several members of chaperone families associate with mHtt. Moreover, levels of Hsp70 and Hsp40 are progressively reduced in brain tissues of HD animal models through a combination of sequestration and transcriptional dysregulation, suggesting that modulation of chaperones could be a therapeutic strategy in HD (Labbadia and Morimoto, 2013). The genetic modulation of chaperone-mediated regulatory pathways in HD has been mainly studied by manipulating Hsp40 and Hsp70 levels and less frequently Hsp90, CHIP and HSF1 levels (Table 1.1.4). Overall, the overexpression or enhancement of chaperones reduced mHtt toxicity in HD cellular and animal models: Hsp40 and CHIP reduced mHtt aggregation and improved HD phenotypes; Hsp90 modulation did not significantly alter HD phenotypes; and contradictory results have been obtained with modulation of HSF1 and Hsp70 (Table 1.1.4).

The chaperone-dependent E3 ubiquitin ligase CHIP was found to interact with mHtt and to induce its ubiquitination and degradation, in cells expressing N-terminal mHtt (Jana et al., 2005). Consistently, CHIP overexpression reduced mHtt aggregation and toxicity in HD cellular and animal models (Jana et al., 2005; Miller et al., 2005; Al-Ramahi et al., 2006).

HSF1 overexpression increased survival and reduced mHtt aggregation in skeletal muscle but not in brains of R6/2 HD mice, where HSF1 expression was not detected (Fujimoto et al., 2005). In contrast, overexpression of HSF1 in a bone-derived cell line increased mHtt aggregation and toxicity. The proposed explanation was that strategies that promote the heat shock response may increase proteotoxic stress, preferentially killing cells with high mHtt expression and this could lead in some cases to an underestimation of mHtt aggregation (Bersuker et al., 2013).

Overexpression of Hsp90 in cell lines showed no effect on mHtt aggregates (Sittler et al., 2001). Hsp90 silencing, however, reduced the levels of mutant Htt in both full-length and N-terminal forms, in cellular models, supporting the hypothesis that Htt is an Hsp90 client protein, and that preventing its cycling with Hsp90 promotes its degradation (Baldo et

al., 2012; Ernst et al., 2014). Further supporting this hypothesis is the fact that both wild-type and mutant N-terminal Htt, and full-length mutant Htt were found to interact with Hsp90 (Baldo et al., 2012).

Hsp40 and Hsp70 overexpression have been studied alone or in combination in different HD models. While Hsp40 overexpression inhibited mHtt aggregation and/or toxicity in cells (Jana et al., 2000; Sittler et al., 2001; Rujano et al., 2007), primary neurons (Zhou et al., 2001), *Xenopus laevis* (Hageman et al., 2010), and mice (Labbadia et al., 2012), Hsp70 overexpression was less efficient in reducing mHtt aggregation than Hsp40. Indeed, HEK293 cells overexpressing Hsp40 exhibited fewer mHtt aggregates than those overexpressing Hsp70 (Zhou et al., 2001). Data suggest that Hsp70 overexpression reduces or does not alter mHtt aggregation, which may be related with the Hsp70 isoform that is modulated. For example, overexpression of Hsc70 decreased mHtt aggregation, while overexpression of inducible Hsp70 did not alter the amount of aggregates in Neuro2a cells expressing mHtt (Jana et al., 2000). Most studies, however, do not specify which Hsp70 isoforms are being modulated. Co-expression of Hsp70 and Hsp40 synergistically reduced mHtt aggregation (Sittler et al., 2001; Rujano et al., 2007), highlighting the coordination between both Hsp70 and Hsp40 chaperones in mediating Htt protein degradation.

Table 1.1.3. Polyglutamine Disorders Summary

Disease (Prevalence [a])	Protein	Normal Q length	Pathogenic Q length	Neuropathology	Main clinical features
HD (5-10/100,000)	Huntingtin	6-34	36-121	Marked neuronal loss in the striatum and cerebral cortex	Chorea, dystonia, bradykinesia, rigidity, cognitive deficits, psychiatric problems
SBMA (1/30,000)	Androgen receptor	9-34	38-62	Degeneration of lower motor neurons in the anterior horn, bulbar region, and dorsal root ganglia	Motor weakness, gynecomastia, testicular atrophy, decreased fertility
SCA1 (1-2/100,000)	Ataxin-1	6-44[b]	39-91	Degeneration of Purkinje cells, cerebellar dentate, inferior olive and red nuclei	Ataxia, slurred speech, spasticity, cognitive impairments
SCA2 (1-2/100,000)	Ataxin-2	14-32	>32	Degeneration of Purkinje and granule neurons	Ataxia, decreased reflexes, retinopathy in infantile cases
SCA3 (1-2/100,000)	Ataxin-3	11-44	45-86	Degeneration of subthalamic nucleus, substantia nigra, dentate nucleus, pontine and cranial nerve nuclei	Ataxia, parkinsonism, spasticity
SCA6 (<1/100,000)	CACNA1A	4-18	19-33	Degeneration of Purkinje cells	Ataxia, dysarthria, nystagmus, tremors
SCA7 (<1/100,000)	Ataxin-7	4-35	>35	Degeneration of retina, cerebellar Purkinje and granule cells	Ataxia, blindness, cardiac failure in infantile cases
SCA17 (unknown)	TBP	25-40	42-66	Degeneration of small neurons in the caudate and putamen, Purkinje cells and frontal and temporal cortex	Ataxia, cognitive decline, seizures, and psychiatric problems
DRPLA (unknown)	Atrophin-1	6-35	49-93	Degeneration of Purkinje cells, cerebral cortex, globus palidus, striatum, dentate, subthalamic and red nuclei	Ataxia, seizures, choreoathetosis, dementia

[a] Worldwide prevalence values from http://www.orpha.net. Orpha numbers: ORPHA399; ORPHA481; ORPHA98755; ORPHA98756; ORPHA98757; ORPHA98758; ORPHA94147; ORPHA98759; ORPHA101.

[b] Normal SCA1 alleles are interrupted by 1-4 CAT sequences, whereas disease-causing alleles are uninterrupted.

Adapted from (Orr and Zoghbi, 2007; Blum et al., 2013).

Table 1.1.4. Genetic Hsp modulation in HD models

Protein	Modulation	Model	Mutant Protein		Survival	Other Outcomes	References
			Aggregation	Levels			
CHIP	Overexpression	**Zebrafish** embryos (Q82)	-	-	↑	improved morphology	(Miller et al., 2005)
		Drosophila (Q128)	-	-	-	↓ retinal degeneration	(Al-Ramahi et al., 2006)
		Cells (Cos-7; Q82)	↓	↑	-	-	(Miller et al., 2005)
		Cells (Neuro2a; Q150)	↓	-	↑	↑ mHtt ubiquitination	(Jana et al., 2005)
		Cells (O23; Q74)	=	↓	-	-	(Rujano et al., 2007)
	Reduction	**Mice** (N171-Q82)	↑	-	↓	↓ DARPP-32 levels in striatum; ↑ motor dysfunction	(Miller et al., 2005)
HSF1	Overexpression	**Mice** (R6/2 without HSF1 overexpression in brain)	↓ (skeletal muscle) = (brain)	-	↑	↓ skeletal muscle damage; = brain atrophy; = weight loss; = paw-clasping	(Fujimoto et al., 2005)
		Cells (U2OS Tet-On; Q91)	↑	-	↓	-	(Bersuker et al., 2013)
Hsp90	Overexpression	**Cells** (COS-1; Q51)	=	-	-	-	(Sittler et al., 2001)
	Reduction	**Cells** (HN10; Q72)	-	↓	-	-	(Baldo et al., 2012)
		Cells (HEK293; Q73)	-	↓	-	-	(Ernst et al., 2014)
Hsp40	Overexpression	**Mice** (R6/2)	↓	↑	-	↓ motor dysfunction; ↑ BDNF levels; = weight loss	(Labbadia et al., 2012)
		Xenopus laevis tadpole (Q119)	↓	-	-	-	(Hageman et al., 2010)
		Primary neurons (striatum; Q120)	↓	-	-	↓ DNA fragmentation	(Zhou et al., 2001)
		Cells (Neuro2a; Q150)	↓	-	↑	-	(Jana et al., 2000)
		Cells (COS-7; Q74)	↑	-	-	-	(Wyttenbach et al., 2000)
		Cells (PC12/SH-SY5Y; Q74)	=	-	-	-	
		Cells (COS-1; Q51)	↓	-	-	-	(Sittler et al., 2001)
		Cells (HEK293; Q120)	↓	-	↑	↓ caspase activity	(Zhou et al., 2001)
		Cells (O23/N2a; Q74)	↓	-	-	-	(Rujano et al., 2007)
		Cells (Q119)	↓	-	↑	-	(Hageman et al., 2010)

Table 1.1.4. *Continued*

	Reduction	**Cells** (HEK293; Q74)	↓	-	-	-	(Hageman et al., 2010)
Hsp70	**Overexpression**	**Mice** (R6/2)	=	-	=	↓ body weight loss; = clasping phenotype; = brain weight loss; = striatum size; = DARPP32 levels	(Hansson et al., 2003)
		Mice (R6/2)	↓ (in early stages)	=	-	↑ weight loss; = motor dysfunction	(Hay et al., 2004)
		Primary neurons (striatum; Q120)	=	-	-	↓ DNA fragmentation	(Zhou et al., 2001)
		Primary neurons (cortex; Q111)	-	-	↑	-	(Tagawa et al., 2007)
		Cells (Neuro2a: Q150)	↓ (Hsc70); = (Hsp72)	-	= (Hsc70)	-	(Jana et al., 2000)
		Cells (COS-7; Q74)	=	-	-	-	(Wyttenbach et al., 2000)
		Cells (HEK293; Q120)	=	-	↑	↓ caspase activity	(Zhou et al., 2001)
		Cells (COS-1; Q51)	↓	-	-	-	(Sittler et al., 2001)
		Cells (O23/N2a; Q74)	=	-	-	-	(Rujano et al., 2007)
		Cells (Q119)	=	-	-	-	(Hageman et al., 2010)
		Cells (SK-N-SH; Q103)	↓	↑	-	-	(Guzhova et al., 2011)
		Cells (Neuro2a; Q150)	↓(Grp78)	-	↑(Grp78)	↓ caspase activity (Grp78)	(Wacker et al., 2009)
	Reduction	**Mice** (R6/2)	↑	-	↓	↑ motor dysfunction; worsened coat appearance; = weight loss	(Tagawa et al., 2007)
		Primary neurons (cerebellum; Q111)	-	-	↓	-	(Jiang et al., 2012)

1.1.3.1.2. Pharmacological modulation of molecular chaperones in HD

Drugs that attempt to rectify the harmful consequences of mHtt (e.g. histone deacetylase inhibitors (Steffan et al., 2001; Oliveira et al., 2006; Guedes-Dias et al., 2015)) have shown promising results. Nevertheless, pharmacological strategies that focus on the causative agent itself, mHtt, via enhanced refolding, and/or increased degradation are expected to provide upstream protection, and are being actively pursued, namely with drugs that modulate molecular chaperones.

Increased expression of molecular chaperones may be achieved pharmacologically by direct activators of HSF1 or by Hsp90 inhibitors. The latter dissociate HSF1 from its cytosolic complexes with Hsp90, allowing HSF1 nuclear translocation and increasing the expression of heat shock proteins (Hay et al., 2004; Fujikake et al., 2008). By destabilizing the Hsp90 chaperone-client protein complexes, Hsp90 inhibitors also promote the degradation of client proteins (Baldo et al., 2012). Studies with Hsp90 inhibitors have found an inhibition of mHtt aggregation and/or a decrease of its toxicity (Table 1.1.5).

HSF1 activators include HSF1A, a benzyl pyrazole-based molecule (Neef et al., 2010) and F1, an unsaturated barbituric acid derivative (Calamini et al., 2012). Both HSF1A and F1 promoted Hsp70 expression and reduced mHtt aggregates in cells expressing mHtt (Neef et al., 2010; Calamini et al., 2012). In contrast, bone-derived cells treated with F1 showed increased mHtt aggregation, however, in this case F1 treatment was performed at least 48h after cellular transfection with Htt exon 1 (Bersuker et al., 2013), a sufficient time for mHtt aggregates to have already formed (Hipp et al., 2012). Together, these findings suggest that the chaperones induced by HSF1 treatment may act on mHtt oligomers but not on the larger aggregates.

Hsp70 has a crucial role in protein folding, transport and degradation (Saibil, 2013) and its overexpression may reduce mHtt aggregation (Sittler et al., 2001). SW02, an Hsp70 ATPase stimulator, decreased mHtt aggregation in PC12 and yeast HD models, increasing soluble mHtt and toxicity in yeast, but unaffecting survival in PC12 cells (Chafekar et al., 2012). Conversely, CE12, an Hsp70 ATPase inhibitor, increased mHtt soluble levels in yeast and increased mHtt aggregation and survival in yeast and PC12 cells, supporting the hypothesis that mHtt aggregates are less toxic than soluble mHtt (Chafekar et al., 2012)

Table 1.1.5. Pharmacological Hsp modulation in polyglutamine disorders

Disease	Target	Pharmacodynamics	Drug	Model	Mutant Protein		Survival	Other outcomes	References
					Aggregation	Levels			
HD	HSF1	Activation	HSF1A	**Cells** (PC12; Q74)	↓	-	↑	-	(Neef et al., 2010)
			F1	**Cells** (PC12; Q74)	↓	=	-	-	(Calamini et al., 2012)
				Cells (U2OS; Q91)	↑	-	-	-	(Bersuker et al., 2013)
	Hsp90	ATPase activity inhibition	Geldanamycin	***Drosophila*** (Q128)	-	-	-	↓ photoreceptor degeneration	(Fujikake et al., 2008)
				Slice (R6/2; hippocampus)	↓	=	-	-	(Hay et al., 2004)
				Cells (COS-1; Q72)	↓	-	-	-	(Sittler et al., 2001)
			Radicicol	***Drosophila*** (Q128)	-	-	-	↓ photoreceptor degeneration	(Fujikake et al., 2008)
				Slice (R6/2; hippocampus)	↓	↑	-	-	(Hay et al., 2004)
			17-DMAG	**Cells** (COS-1; Q72)	↓	-	-	-	(Herbst and Wanker, 2007)
			17-AAG	***Drosophila*** (Q128)	-	-	-	↓ photoreceptor degeneration	(Fujikake et al., 2008)
				Cells (COS-1; Q72)	↓	-	-	-	(Herbst and Wanker, 2007)
			NVP-HSP990	**Mice** (R6/2)	↓	-	-	Improved rotarod performance; ↑ brain weight; = grip strength; = exploratory activity; = weight loss	(Labbadia et al., 2011)
			NVP-AUY922	**Cells** (HN10; Q72/ ES; Q150)	-	↓	-	-	(Baldo et al., 2012)
	Hsp70	ATPase activity stimulation	SW02	**Yeast** (Q46)	↓	↑	↓	-	(Chafekar et al., 2012)
				Cells (PC12; Q103)	↓	-	=	-	
		ATPase activity inhibition	CE12	**Yeast** (Q46)	↑	↓	↑	-	
				Cells (PC12; Q103)	↑	-	↑	-	
SBMA	Hsp90	ATPase activity inhibition	Geldanamycin	**Cells** (MEFs; Q112)	↓	-	-	-	(Thomas et al., 2006)
				Cells (MEFs/MN-1; Q112)	-	↓	-	-	(Morishima et al., 2008)

Table 1.1.5. *Continued*

			Radicicol	**Cells** (MEFs; Q112)	↓	-	-	-	(Thomas et al., 2006)
			17-DMAG	**Mice** (Q97)	↓	↓	↑	↓ motor dysfunction; ↓ weight loss	(Tokui et al., 2009)
				Cells (SH-SY5Y; Q97)	-	↓	-	-	
			17-AAG	**Mice** (Q97)	↓	↓	↑	↓ motor dysfunction; ↓ weight loss	(Waza et al., 2005)
				Cells (SH-SY5Y; Q97)	-	↓	-	-	
				Cells (SH-SY5Y; Q97)	-	↓	-	-	(Tokui et al., 2009)
				Cells (NSC34; Q48)	↓	↓	-	-	(Rusmini et al., 2011)
	Hsp70	HSF-1 binding inhibition	GGA	**Mice** (Q97)	-	↓	↑	↓ motor dysfunction; ↓ weight loss	(Katsuno et al., 2005)
				Cells (SH-SY5Y; Q97)	=	↓	↑	-	
		ATPase activity inhibition	Methylene blue	**Cells** (HeLa; Q112)	-	↑	-	-	(Wang et al., 2010)
		ATPase activity inhibition / ADP-bound form stabilization	YM-1	***Drosophila*** (Q52)	-	-	-	↓ eye degeneration; ↑ eclosion	(Wang et al., 2013)
				Cells (PC12; Q112)	↓	↓	-	-	
SCA3	**HSF1**	Activation	HSF1A	***Drosophila*** (Q78)	-	-	-	↓ eye degeneration	(Neef et al., 2010)
	Hsp90	ATPase activity inhibition	Geldanamycin	***Drosophila*** (Q78)	-	-	-	↓ eye degeneration	(Fujikake et al., 2008)
				Cells (MN-1; Q78)	-	=	-	-	(Morishima et al., 2008)
			Radicicol	***Drosophila*** (Q78)	-	-	-	↓ eye degeneration	(Fujikake et al., 2008)
			17-DMAG	**Mice** (Q135)	↓	↓	-	↓ motor dysfunction; = body weight; ↑ beclin-1 and LC3-II levels	(Silva-Fernandes et al., 2014)
			17-AAG	***Drosophila*** (Q78)	↓	=	↑	↓ eye degeneration	(Fujikake et al., 2008)
				Drosophila (Q78)	-	-	-	↓ eye degeneration	(Neef et al., 2010)
	Hsp70	HSF1 binding inhibition	GGA	***Drosophila*** (Q78)	-	-	-	= eye degeneration	(Fujikake et al., 2008)
				Cells (HEK293; Q75)	↓	=	-	-	(Lin et al., 2014)

Table 1.1.5. *Continued*

				Cells (SH-SY5Y; Q75)	↓	-	-	↑ neurite outgrowth	
		ATPase activity inhibition	Azure C	**Cells** (HEK293; Q22/71)	-	↑	-	↑ levels of wild type ataxin-3	(Gao et al., 2011)
	-	-	Indole/NC001-8	**Cells** (HEK293; Q75)	↓	=	-	↓ caspase activity; ↑ LC3-II levels; ↓ ROS production	(Lin et al., 2014)
				Cells (SH-SY5Y; Q75)	↓	-	-	↑ neurite outgrowth	
SCA17	**Hsp70**	HSF1 binding inhibition	GGA	**Cells** (SH-SY5Y; Q79)	↓	-	-	-	(Kung et al., 2014)
	-	-	Indole/NC001-8	**Slice** (cerebellum; Q109)	↓	-	-	-	
				Primary neurons (cerebellum; Q109)	↓	-	-	↑ neurite outgrowth	
				Cells (SH-SY5Y; Q79)	↓	-	-	-	

1.1.3.2. Spinal and bulbar muscular atrophy

SBMA, also known as Kennedy's disease, is caused by polyQ expansion in the androgen receptor (AR) (Katsuno et al., 2012) (Table 1.1.3). The AR mediates the effects of androgens, being expressed in sexual and non-reproductive organs, such as the skeletal muscle, and the central nervous system (Katsuno et al., 2012). AR-androgen complexes formed in the cytoplasm translocate to the nucleus where they activate gene transcription (Orr, 2012b). Mutant AR forms insoluble aggregates in the nucleus and cytoplasm (Gatchel and Zoghbi, 2005), however, nuclear localization seems critical for toxicity since mutations in the nuclear localization signal or addition of nuclear export signals abolish mutant AR toxicity (Takeyama et al., 2002; Montie et al., 2009). Still, ligand-dependent nuclear localization *per se* is insufficient for mutant AR-induced neurodegeneration, as DNA binding is also required (Nedelsky et al., 2010).

As in HD, the pathogenesis of SBMA includes transcriptional dysregulation and mitochondrial dysfunction. Mutant AR decreases the expression of genes required for neuronal survival (Sopher et al., 2004; Katsuno et al., 2010), and its N-terminal fragments were found to activate BAX-dependent apoptosis in neurons, stimulating cytochrome c release from mitochondria (Young et al., 2009). Further, mutant AR expression in mammalian cells was found to increase reactive oxygen species, depolarize mitochondria and to decrease transcription of genes associated with mitochondrial function, such as PGC-1β, TFAM, PPARγ, ND1, SOD2 (Ranganathan et al., 2009).

1.1.3.2.1. Genetic modulation of molecular chaperones in SBMA

The AR is a classic Hsp90 client protein and the genetic modulation of chaperones in the context of SBMA has been performed by overexpression of CHIP, Hip, Hsp40 and Hsp70 in cell lines and less frequently in animal models. Available studies suggest that overexpression of chaperones decreases mutant AR levels, aggregates and toxicity (Table 1.1.6).

CHIP overexpression enhanced mutant AR ubiquitination and degradation, decreasing mutant AR levels in cells (Adachi et al., 2007; Morishima et al., 2008) and in SBMA transgenic mice, where it also decreased mutant AR aggregation and ameliorated motor symptoms (Adachi et al., 2007). Similarly, Hip overexpression decreased mutant AR aggregation by increasing its degradation in cells (Howarth et al., 2009; Wang et al., 2013).

Hsp40 or Hsp70 overexpression decreased mutant AR aggregate formation and increased the survival of SBMA cell models (Table 1.1.5). Hsp70 overexpression in SBMA

transgenic mice ameliorated motor function and decreased aggregation and levels of mutant AR. (Adachi et al., 2003). Overexpression of Hsp40 and Hsp70 likely decreases the aggregation of mutant AR by promoting its degradation via the UPS. Alternatively, both Hsp40 and Hsp70 also induce mutant AR solubilization, since their overexpression decreased mutant AR aggregation and increased its soluble form in presence of lactacystin, a proteasome inhibitor (Bailey et al., 2002). Co-expression of Hsp70 and Hsp40 presents synergic effects on reduction of mutant AR aggregate formation and on cell survival, reflecting that Hsp70 and Hsp40 act together in chaperoning misfolded AR (Kobayashi et al., 2000; Bailey et al., 2002).

Table 1.1.6. Genetic Hsp modulation in SBMA models

Protein	Modulation	Model	Mutant Protein		Survival	Other Outcomes	References
			Aggregation	Levels			
CHIP	Overexpression	**Mice** (Q97)	↓	↓	↑	↓ motor dysfunction; ↓ muscle atrophy; ↓ weight loss	(Adachi et al., 2007)
		Cells (SH-SY5Y; Q65)	-	↓	-	-	
		Cells (MN-1; Q112)	-	↓	-	-	(Morishima et al., 2008)
HIP	Overexpression	***Drosophila*** (Q52)	-	-	-	↑ eclosion	(Wang et al., 2013)
		Cells (N2a; Q51)	↓	-	-	-	(Howarth et al., 2009)
		Cells (HeLa; Q112)	↓	↓	-	-	(Wang et al., 2013)
Hsp40	Overexpression	**Cells** (HeLa; Q48)	↓	-	-	-	(Stenoien et al., 1999)
		Cells (Neuro2a: Q97)	↓	-	↑	-	(Kobayashi et al., 2000)
		Cells (MN hybrid cell; Q112)	↓	-	-	-	(Bailey et al., 2002)
		Cells (N2a; Q51)	↓	-	-	-	(Howarth et al., 2007)
		Cells (N2a; Q51)	↓	-	-	-	(Howarth et al., 2009)
		Cells (Q72)	↓	-	-	-	(Hageman et al., 2010)
Hsp70	Overexpression	**Mice** (Q97)	↓	↓	-	↓ motor dysfunction; ↓ weight loss	(Adachi et al., 2003)
		Cells (Neuro2a: Q97)	↓	-	↑	-	(Kobayashi et al., 2000)
		Cells (MN hybrid cell; Q112)	↓	-	-	-	(Bailey et al., 2002)
		Cells (N2a; Q51)	↓	-	-	-	(Howarth et al., 2007)
		Cells (N2a; Q51)	↓	-	-	-	(Howarth et al., 2009)

1.1.3.2.2. Pharmacological modulation of molecular chaperones in SBMA

Several drugs targeting Hsp90 and Hsp70 chaperones have been tested in cellular and animal models of SBMA (Table 1.1.5). As with mHtt in HD models, Hsp90 inhibitors reduced aggregation and/or soluble levels of mutant AR, decreasing its toxicity in SBMA models (Katsuno et al., 2005; Waza et al., 2005; Tokui et al., 2009). The geldanamycin derivative 17-DMAG was 3-fold more potent than 17-AAG in reducing levels of mutant AR in SH-SY5Y cells (Tokui et al., 2009). Proteasomal inhibition with MG-132 blocked the reduction of mutant AR levels induced by 17-AAG and 17-DMAG. In contrast, these Hsp90 inhibitors decreased mutant AR even when Hsp70 induction was blocked by protein synthesis inhibition with cycloheximide or by small interfering RNA. These results indicate that the effects of these Hsp90 inhibitors are highly dependent on the UPS, rather than on Hsp70 induction (Waza et al., 2005; Tokui et al., 2009). Autophagy also seems involved in the reduction of mutant AR after 17-AAG treatment. Indeed, 17-AAG may promote activation of the autophagy pathway, as indicated by the associated increases in LC3 mRNA and LC3-II protein levels in immortalized motor neurons. In addition, when autophagy was blocked with 3-methyladenine or with small-hairpin RNA against LC3, 17-AAG could not increase degradation of mutant AR (Rusmini et al., 2011).

GGA has been shown to induce the expression of molecular chaperones in various tissues, including the central nervous system, via disruption of the Hsp70-HSF1 complex, thus allowing HSF1 activation (Fujiki et al., 2003; Otaka et al., 2007; Sinn et al., 2007). GGA did not alter the expression of the constitutive Hsc70, but up-regulated expression of Hsp70 and Hsp90 in cells expressing mutant AR (Katsuno et al., 2005). GGA also decreased mutant AR levels and increased the survival of SBMA cellular and mouse models (Katsuno et al., 2005).

Methylene blue, which was identified in a high-throughput screen as an Hsp70 inhibitor (Jinwal et al., 2009), promoted the accumulation of mutant AR in HeLa cells. Since methylene blue may influence several cellular processes, the experiments were repeated in the presence of Hsp70 overexpression, and the associated loss of methylene blue effects on mutant AR indicates that they were mediated by Hsp70 inhibition (Wang et al., 2010).

YM-1 stabilizes Hsp70 in its ADP-bound state, mimicking the co-chaperone Hip and enhancing binding of Hsp70 to substrates. In a PC12 cell model of SBMA, YM-1 decreased the levels of mutant AR through increased degradation by the UPS. Importantly, the knockdown of Hsp70 reduced the effects of YM-1, implicating Hsp70 as its critical cellular target (Wang et al., 2013).

1.1.3.3. Spinocerebellar ataxias and dentatorubral-pallidoluysian atrophy

The SCA family comprises more than 35 genetically different types of progressive neurodegenerative disorders, six of which are polyQ expansion disorders (Jacobi et al., 2015). SCA1, 2, 3 and 7 are caused by polyQ expansion in ataxin-1, 2, 3 and 7, respectively, whereas SCA6 and SCA17 result from polyQ expansions in the α1A subunit of the voltage-dependent calcium channel Cav2.1 (CACNA1A) and in the TATA-box binding protein (TBP), respectively (Orr, 2012a) (Table 1.1.3). SCAs and DRPLA share many clinical and pathological features (Table 3), in fact, DRPLA patients with mildly expanded polyQ in the affected protein (atrophin-1; (Shen and Peterson, 2009)) tend to exhibit pure cerebellar symptoms, such as ataxia (Tsuji, 2012). Many of the ataxia-causing proteins are reported to share interacting partners, suggesting that the disease phenotypes shared among these disorders may result from common molecular pathways (Lim et al., 2006). Indeed, as will be described below, transcriptional dysregulation is a common feature to all of these disorders.

Ataxin-1 was reported to interact with RNA and several regulators of transcription, indicating its role in the regulation of gene expression (Yue et al., 2001; Lim et al., 2008; de Chiara et al., 2009). Studies in transgenic animal models showed that expanded polyQ in ataxin-1 induced a dysregulation of the expression of genes critical for cerebellar development (Serra et al., 2004; Serra et al., 2006; Crespo-Barreto et al., 2010). Importantly, nuclear localization of the mutant protein seems to be required for toxicity (Klement et al., 1998) and nuclear aggregates are common in SCA1 (Rub et al., 2013).

Ataxin-2 is thought to play a role in post-transcriptional and translation regulation (Magana et al., 2013). Mice expressing ataxin-2 with a polyQ expansion showed deficits in the expression of Purkinje cells-specific genes (Hansen et al., 2013; Dansithong et al., 2015), which were associated with translation dysregulation (Dansithong et al., 2015). In contrast with SCA1, nuclear localization or aggregates formation of the expanded ataxin-2 are thought unnecessary for the pathogenesis of SCA2 (Huynh et al., 2000).

Ataxin-3 has been associated with protein homeostasis and transcriptional regulation (Costa Mdo and Paulson, 2012). Ataxin-3 has ubiquitin-protease activity and up to three ubiquitin-binding motifs, being thought to directly regulate ubiquitination-dependent degradation pathways (Li et al., 2015). Additionally, ataxin-3 was shown to interact with the co-chaperone and E3 ligase CHIP and suggested to promote the turnover of CHIP substrates (Scaglione et al., 2011). The expansion of the polyQ tract in ataxin-3 may alter the dynamics of ataxin-3 and CHIP interaction, targeting CHIP for degradation. In fact, expanded ataxin-3 presents an increased affinity for CHIP, and decreased levels of CHIP were found in a SCA3 mouse model (Scaglione et al., 2011). Consistently with its function

in transcription, expression of mutant ataxin-3 in SCA3 transgenic mice altered the expression of several genes including those involved in the heat shock response (Chou et al., 2008). Although both cytoplasmic and nuclear aggregates have been described in SCA3 brains (Costa Mdo and Paulson, 2012), the nucleus is thought to be the principal site of SCA3 pathogenesis (Bichelmeier et al., 2007).

CACNA1A, the protein mutated in SCA6, integrates the Cav2.1 calcium channel that is responsible for transmission initiation at fast synapses (Orr, 2012a). While there is lack of support for changes in Cav2.1 channel function in SCA6 (Rub et al., 2013; Giunti et al., 2015), this disease has been associated with transcriptional dysregulation in Purkinje cells (Du et al., 2013), being noteworthy that mutant CACNA1A aggregation preferentially occurs in the cytoplasm and rarely in the nucleus of Purkinje cells (Ishiguro et al., 2010).

Ataxin-7 constitutes a subunit of the TFTC (TATA-binding protein-free TAF-containing complex) and the STAGA (SPT3/TAF GCN5 complex) complexes, both of which are crucial for gene transcription (Rub et al., 2013). Not surprisingly, mutant ataxin-7 induced transcriptional dysregulation in SCA7 transgenic models (Yoo et al., 2003; Helmlinger et al., 2006; Chou et al., 2010), and the disease associates with nuclear aggregates of the polyQ-expanded protein (Seidel et al., 2012).

TBP, the protein involved in SCA17, is an essential component of the transcriptional initiation complex (Vannini and Cramer, 2012), directing it to DNA via binding to the TATA-box (Sainsbury et al., 2015). Studies with SCA17 transgenic mice indicate that polyQ expansion in TBP alters its ability to bind DNA and transcriptional regulators, suggesting that transcriptional dysregulation contributes to SCA17 pathogenesis (Friedman et al., 2007; Friedman et al., 2008; Shah et al., 2009; Huang et al., 2011). In SCA17, mutant TBP accumulates in intranuclear aggregates (Seidel et al., 2012).

Atrophin-1, the protein involved in DRPLA, functions as a transcription regulator (Shen and Peterson, 2009). In *Drosophila* models (Zhang et al., 2002) and brains of transgenic mice (Suzuki et al., 2012), polyQ expansion in atrophin-1 was shown to induce transcriptional dysregulation. Both patients and DRPLA animal models show nuclear accumulation of an N-terminal fragment of mutant atrophin-1, which forms aggregates and is associated with toxicity (Nucifora et al., 2003; Seidel et al., 2012).

1.1.3.3.1. Genetic modulation of molecular chaperones in SCAs and DRPLA

Chaperone-associated genetic modulations in SCAs and DRPLA models include the overexpression of CHIP, Hsp40 and Hsp70, which were found protective by decreasing mutant proteins levels, aggregation, or toxicity (Table 1.1.7). Overexpression of the

chaperone-dependent E3 ubiquitin ligase CHIP decreased mutant protein levels in cellular and *Drosophila* models of SCA1 and SCA3 (Jana et al., 2005; Al-Ramahi et al., 2006; Choi et al., 2007; Morishima et al., 2008; Williams et al., 2009), likely due to increased degradation via the UPS since the ubiquitination levels of mutant protein were increased (Jana et al., 2005; Al-Ramahi et al., 2006). As observed for mHtt aggregation (Jana et al., 2005; Miller et al., 2005), CHIP overexpression decreased mutant ataxin-3 aggregation in cells (Jana et al., 2005). In contrast with these results, CHIP overexpression increased mutant ataxin-1 aggregation in cellular models, suggesting that CHIP may exert different effects depending on its client protein (Choi et al., 2007). Additionally, CHIP modulation was found to alter the levels of unexpanded ataxin-1 and -3 proteins in cells (Table 1.1.7), indicating that CHIP can also regulate the degradation of wild-type forms of these proteins (Choi et al., 2007; Gao et al., 2011). In agreement with the protective effects of CHIP overexpression, CHIP reduction increased mutant ataxin-3 aggregation and the severity of the phenotype in SCA3 transgenic mice (Williams et al., 2009).

Hsp40 overexpression decreased mutant protein aggregation in SCA1, SCA3 and DRPLA cellular models (Cummings et al., 1998; Chai et al., 1999; Fujimoto et al., 2005; Hageman et al., 2010; Gao et al., 2011). Hsp40 overexpression decreased mutant ataxin-3 aggregation while increasing its levels in cells, prompting the hypothesis that Hsp40 may bind mutant ataxin-3 and delay its degradation (Gao et al., 2011). Hsp70 overexpression decreased mutant protein aggregation in SCA3, SCA7 and DRPLA cellular models (Helmlinger et al., 2004; Fujimoto et al., 2005; Lin et al., 2014). In a SCA3 *Drosophila* model, overexpression of Hsp40 or Hsp70 reduced mutant ataxin-3 induced toxicity. Co-overexpression of Hsp40 and Hsp70 synergistically reduced toxicity while decreasing aggregates and increasing soluble ataxin-3 levels, thus associating reduced toxicity with altered solubility properties of mutant ataxin-3 (Chan et al., 2000). As observed for CHIP, Hsp40 and Hsp70 were shown to also regulate the levels of wild-type ataxin-3 in cells (Gao et al., 2011) (Table 1.1.7). While Hsp70 overexpression improved Purkinje neuron morphology and motor function in SCA1 mice (Cummings et al., 2001), its overexpression had no effect on mutant ataxin-7 toxicity in SCA7 mice (Helmlinger et al., 2004). Concerning SCA17, overexpression of the constitutive Hsc70 ameliorated neuropathology in transgenic mice (Yang et al., 2014).

Table 1.1.7. Genetic Hsp modulation in SCAs and DRPLA models

Disease	Protein	Modulation	Model	Mutant Protein		Survival	Other Outcomes	References
				Aggregation	Levels			
SCA1	CHIP	Overexpression	***Drosophila*** (Q2/30/82)	-	↓	-	↓ eye degeneration	(Al-Ramahi et al., 2006)
			Cells (HeLa; Q82)	-	-	-	↑ mutant ataxin-1 ubiquitination	
			Cells (BOSC23; Q30/82)	↑	↓	-	↑ aggregation and ↓ levels of wild type ataxin-1; ↑ ubiquitination of both wild-type and mutant ataxin-1 in insoluble fraction	(Choi et al., 2007)
	Hsp40	Overexpression	**Cells** (HeLa; Q92)	↓	-	-	-	(Cummings et al., 1998)
	Hsp70	Overexpression	**Mice** (B05)	=	-	-	improved motor function; improved purkinje cell morphology	(Cummings et al., 2001)
SCA3	HSF1	Reduction	***Drosophila*** (Q78)	-	-	-	↑ eye degeneration	(Fujikake et al., 2008)
	CHIP	Overexpression	**Cells** (Neuro2a; Q80/130)	↓	↓	-	↑ mutant ataxin-3 ubiquitination;	(Jana et al., 2005)
			Cells (BOSC23; Q73)	-	↓	-	-	(Choi et al., 2007)
			Cells (HEK293/MN-1; Q78)	-	↓	-	-	(Morishima et al., 2008)
			Cells (M17; Q80)	-	↓	-	-	(Williams et al., 2009)
			Cells (HEK293; Q80)	-	=	-	-	
			Cells (HEK293; Q22)	-	-	-	↑ ubiquitination and ↓ levels of wild type ataxin-3	(Gao et al., 2011)
		Reduction	**Mice** (Q71-B)	↑	-	-	↑ motor dysfunction; ↑ nuclear localization of mutant ataxin-3	(Williams et al., 2009)
			Cells (HEK293; Q22)	-	-	-	↑ levels of wild type ataxin-3	(Gao et al., 2011)
	Hsp40	Overexpression	***Drosophila*** (Q78)	-	-	↑	↓ eye degeneration	(Chan et al., 2000)
			Cells (Q82)	↓	-	-	-	(Hageman et al., 2010)
			Cells (HEK293; Q22/71)	↓	↑	-	↑ ubiquitination and levels of wild type ataxin-3	(Gao et al., 2011)
			Cells (COS7/PC12; Q80)	↓	-	-	-	(Chai et al., 1999)

Table 1.1.7. *Continued*

	Hsp70	**Overexpression**	***Drosophila*** (Q78)	-	-	↑	↓ eye degeneration	(Chan et al., 2000)
			Cells (COS7/PC12; Q80)	=	-	-	-	(Chai et al., 1999)
			Cells (HEK293; Q22)	-	-	-	↓ levels of wild type ataxin-3	(Gao et al., 2011)
			Cells (HEK293; Q75)	↓	-	-	-	(Lin et al., 2014)
SCA6	**Hsp70**	**Reduction**	**Cells** (HEK293; Q24)	-	-	=	-	(Li et al., 2009)
SCA7	**Hsp40**	**Overexpression**	**Mice** (R7E; Hsp overexpression exclusively in rod photoreceptors)	-	-	-	= rod photoreceptors dysfunction	(Helmlinger et al., 2004)
			Cells (HEK293; Q128)	=	-	-	-	
	Hsp70	**Overexpression**	**Mice** (R7E; Hsp overexpression exclusively in rod photoreceptors)	-	-	-	= rod photoreceptors dysfunction	(Helmlinger et al., 2004)
			Cells (HEK293; Q128)	↓	-	-	-	
SCA17	**Hsp70**	**Overexpression**	**Mice** (Q105)	-	-	-	↓ degeneration of purkinje cells (Hsc70)	(Yang et al., 2014)
			Cells (PC12; Q105)	-	-	-	↓ neurite outgrowth deficit (Hsc70)	
DRPLA	**HSF1**	**Overexpression**	**Cells** (HeLa; Q81)	↓	-	-	-	(Fujimoto et al., 2005)
	Hsp40	**Overexpression**	**Cells** (HeLa; Q81)	↓	-	-	-	
	Hsp70	**Overexpression**	**Cells** (HeLa; Q81)	↓	-	-	-	

1.1.3.3.2. Pharmacological modulation of molecular chaperones in SCAs and DRPLA

Concerning SCAs and DRPLA, and as far as we could find in the literature, pharmacological modulation of chaperones has only been tested in SCA3 and SCA17 models, where Hsp90 inhibitors or inducers of chaperone expression (GGA, indole or NC001-8) were found protective by decreasing mutant protein aggregation and associated toxicity, in contrast with Hsp70 ATPase inhibition (azure C) (Table 1.1.5).

Hsp90 inhibition with geldanamycin, radicicol or 17-AAG in Drosophila SCA3 models decreased mutant ataxin-3 associated toxicity (Fujikake et al., 2008; Neef et al., 2010), and 17-AAG treatment also decreased mutant ataxin-3 aggregation and increased survival (Fujikake et al., 2008). In SCA3 mice, Hsp90 inhibition with 17-DMAG improved motor function, while decreasing mutant ataxin-3 aggregation and levels, possibly via autophagy activation since 17-DMAG treatment increased both beclin-1 and LC3-II levels (Silva-Fernandes et al., 2014).

Treatment with GGA decreased mutant protein aggregation in SCA3 and SCA17 cells (Kung et al., 2014; Lin et al., 2014), but had no effect on mutant ataxin-3 toxicity in SCA3 Drosophila (Fujikake et al., 2008). Indole and its derivate NC001-8 were recently tested in SCA models. Although the mechanisms by which these compounds induce chaperone expression remain uncertain, both decreased mutant protein aggregation in SCA3 and SCA17 cells (Kung et al., 2014; Lin et al., 2014), being reported to enhance autophagy in SCA3 cells (Lin et al., 2014).

The inhibition of Hsp70 ATPase activity with azure C increased levels of both normal and mutant ataxin-3 in cells, consistent with the hypothesis that Hsp70 can regulate the degradation of wild-type ataxin-3 (Gao et al., 2011).

1.1.3.4. Insights from other neurodegenerative disorders

In addition to polyQ diseases, Hsp70 modulators have been studied in the context of other neurodegenerative disorders. For instance, the Hsp70 ATPase activity stimulators SW02 and 115-7c were assessed for their effect on the proteostasis of tau and α-synuclein, which are proteins know to misfold and accumulate in Alzheimer's and Parkinson's diseases, respectively. In HeLa cells, both compounds were found to increase tau levels (Jinwal et al., 2009). While SW02 had no effect on α-synuclein levels in HeLa cells (Jinwal et al., 2009), 115-7c decreased α-synuclein aggregation in neuroglioma cells (Kilpatrick et al., 2013). Inhibitors of the Hsp70 ATPase activity were also studied for their potential in the

regulation of tau (Jinwal et al., 2009). Contrasting with the observed effects in polyQ models, in which methylene blue and azure C increased protein levels of mutant AR and mutant ataxin-3, respectively, treatment with either of these compounds decreased both wild-type and mutant tau levels in HeLa cells and mice brain (Jinwal et al., 2009). Additionally, neither methylene blue nor azure C altered α-synuclein levels in HeLa cells (Jinwal et al., 2009). YM-1 and its blood brain barrier permeable neutral analog YM-8 were also found to reduce tau levels in HeLa cells and brain slices from tau transgenic mice (Abisambra et al., 2013; Miyata et al., 2013). As observed for SBMA models (Wang et al., 2013), the effect of YM-1 was blocked by proteasomal inhibition with epoximicin, suggesting that treatment with YM-1 promotes tau degradation via the UPS (Abisambra et al., 2013). Taken together with data from polyQ disease models, the emerging picture is that chaperone modulators, and those of Hsp70 in particular, may lead to different effects on the proteostasis of different mutant and wild-type client proteins.

1.1.3.5. Pharmacological targeting of Hsp70: inhibition *versus* activation

Collectively, data from both genetic and pharmacological interventions generally support chaperone modulation as a potential therapeutic approach in polyQ disorders, while also evidencing that the chaperone system may exert different effects depending on the nature of the client protein. Importantly, although genetic interventions support Hsp70 activation as a promising approach for polyQ disorders, the pharmacological modulation of Hsp70 has proven to be more complex.

Two molecules known to inhibit Hsp70 ATPase activity presented different effects on mutant AR levels in SBMA cells (methylene blue increased whereas YM-1 decreased AR levels) (Wang et al., 2010; Wang et al., 2013). One explanation for these distinct results may lay in the detailed mechanisms by which the compounds affect the biochemistry of Hap70. In fact, YM-1 not only inhibits Hsp70 ATPase activity, but it also stabilizes the ADP-bound conformation of Hsp70. This is relevant because the binding affinity of the SBD is highly dependent on the state of the NBD: Hsp70 in an ATP-bound form has poor affinity for client proteins, whereas Hsp70 in an ADP-bound form has high affinity for client proteins (Saibil, 2013). Different Hsp70 modulators may thus evoke different effects on proteostasis depending on their Hsp70 binding site and the conformation state that they promote. As such, modulators that stabilize the ADP-bound conformation, such as YM-1, are expected to enhance Hsp70 affinity to client proteins, inhibiting protein aggregation and allowing Hsp70-dependent degradation. In contrast, modulators that favor the ATP-bound

conformation are expected to promote client release, and thus facilitate mutant protein aggregation.

The mechanisms that regulate the probability of the Hsp70 complex to allow degradation or refolding of a protein are incompletely understood. Several factors, such as the nature of a specific client, its conformational state, and the availability of downstream chaperones and degradation machineries are likely to contribute for that probability.

1.1.4. Outlook

The modulation of molecular chaperones is emerging as a potential therapeutic approach in polyQ disorders. Although genetic approaches have been valuable tools to identify the pathways that modulate the toxicity of polyQ-expanded proteins, the translation of these findings to the clinical practice would benefit from pharmacological agents with the ability to modify disease progression. Hsp90 inhibitors include a variety of molecules that have been extensively studied in the context of polyQ disorders with promising results. However, when considering the long-term use of such inhibitors it is important to have in mind that Hsp90 interacts with not-yet-unfolded client proteins, preserving their stability and activity. Long-term treatment with Hsp90 inhibitors may thus lead to functional disruption and unspecific degradation of multiple Hsp90 client proteins. Taking this into account, the ideal approach might be to promote the degradation of not properly folded proteins that are no longer cycling with Hsp90. In principle, this may be achieved with drugs that modulate Hsp70, promoting Hsp70-dependent degradation. Current limitations are that all the available Hsp70 modulators lack selectivity for individual Hsp70 family members, increasing the chances of disrupting essential functions of multiple Hsp70 isoforms, including the Hsp70 present in the mitochondria. As such, in addition to the development of selective Hsp70 inhibitors, future studies in polyQ models should include the effects of Hsp70 modulators on mitochondrial Hsp70 functions, including mitochondrial protein import and folding. Moreover, given that the consequences of modulating Hsp70 activity seem to depend on the nature of the specific client protein, it currently seems unlikely that the same Hsp70 modulator will provide therapeutic benefits for all polyQ disorders. Furthermore, since Hsp70 may not only influence the levels of soluble mutant protein but also act upon aggregates, it will be important to clarify the relative pathological role of such protein species and how these are affected by different Hsp70 modulators.

Conflict of interest

The authors declare they have no conflict of interest

CHAPTER II

OBJECTIVES

2. Objectives

The levels of different Hsp are reduced or unaltered in models of HD, which indicates a possible impairment of the HSR (Yamanaka et al., 2008; Chafekar and Duennwald, 2012; Neueder et al., 2017; Scior et al., 2018). Hsp70 is a main effector of the proteostasis network, acting in client protein by redirecting them for either folding or degradation, or by promoting its disaggregation (Mayer, 2013; Karagoz and Rudiger, 2015; Nillegoda et al., 2015; Nillegoda et al., 2018). The overexpression of Hsp70 alone or in combination with Hsp40 has been tested in different HD models with promising results (Reis et al., 2017). Moreover, the combined addition of Hsp70, Hsp40 and Hsp110 family members reversed the aggregation of mHtt in vitro (Scior et al., 2018). Pharmacological modulation of Hsp70 with YM-1 decreased the levels of mutant AR and tau in models of SBMA and tauopathies, respectively, supporting its potential in neurodegenerative disorders characterized by the accumulation of misfolded proteins (Abisambra et al., 2013; Miyata et al., 2013; Wang et al., 2013; Young et al., 2016). However, the mechanisms that regulate Hsp70 action are not completely understood and several factors, such as the nature of the client protein, its conformational state, and the availability of downstream chaperones and degradation machineries, probably affect the outcome of modulating Hsp70, justifying the study of the potential of YM-1 on HD.

2.1. Open questions

Do different mHtt species differentially affect the HSR?

Does the chaperone machinery act differentially on the different Htt species?

Does mHtt aggregation trigger the formation of a trimeric disaggregase complex composed by Hsp70, Hsp40 and Hsp110?

Is Hsp70 modulation with YM-1 also protective in HD models?

2.2. General aims

The main aims of this work were:

- To characterize the proteostasis of different species of Htt, focusing on how it relates with the molecular chaperone machinery
- To evaluate the potential of Hsp70 modulation to alter Htt proteostasis and toxicity

2.3. Specific aims

The specific aims were the following:

- To assess the levels of diffuse and aggregated Htt in different HD cellular models expressing either the full-length or the N-terminal form of Htt
- To assess the levels and localization of different Hsp in the different HD models
- To investigate whether Hsp70 modulation through induction of the HSR or with the Hsp70 ATPase activity inhibitor YM-1 alters full-length Htt proteostasis
- To investigate whether Hsp70 modulation with YM-1 alters N-terminal Htt proteostasis and cell death
- To investigate whether Hsp70 modulation with YM-1 affects mitochondrial function in N-terminal Htt expressing cells

CHAPTER III

MATERIALS AND METHODS

3. Materials and Methods

3.1. PC12 cell culture

Rat pheochromocytoma cells, PC12 cells, with inducible expressing of full-length human Htt containing 23 (wild-type), 73 or 145 (mutant) glutamine repeats with an RFP (Red Fluorescent Protein) tag at the C-terminus (RFP-FL-HttQ23; -HttQ73; -HttQ145; Coriell Institute) were grown in DMEM high glucose (Gibco) supplemented with 15% horse serum, 2.5% fetal bovine serum, 0.2mg/ml geneticin (Gibco), 0.2mg/ml zeocin (Invivogen) and 1% penicillin/streptomycin, at 37°C in 5% CO_2. To induce Htt expression, cells were incubated with 5µM ponasterone A (Sigma-Aldrich) in 0.1% dimethyl sulfoxide (DMSO) for the indicated time periods.

3.2. U2OS cell culture and transfection

Human osteosarcoma cells, U2OS cells, were grown in DMEM without glucose (Gibco) supplemented with 10mM galactose, 2mM glutamine, 5mM HEPES, 1mM sodium pyruvate, 10% fetal bovine serum and 1% penicillin/streptomycin, at 37°C in 5% CO_2. For transfection, cells were incubated with Opti-MEM media (Gibco), containing 0.5-2.5µg plasmid DNA per coverslip/well and Lipofectamine LTX and Plus reagent (Invitrogen) in a 1:1 ratio to DNA (0.5-2.5µl) for 45 min at 37ºC. After incubation, cells were washed once with DMEM in order to remove the transfection complexes and left to grown in their appropriate supplemented media.16-18h after transfection, cells were treated as indicated.

3.3. Fluorescence activated cell sorting

PC12 cells were resuspended in sorting buffer containing 1mM EDTA, 25mM HEPES and 2% fetal bovine serum, prepared in phosphate-buffered saline buffer (PBS). After filtration, cells were sorted based on their granularity/complexity, size and RFP-tag fluorescence in a concentration of 1.0 x 10^7 cells/mL and collected in PC12 complete media containing 2% penicillin/streptomycin. Uninduced cells were used as controls. The RFP negative and the RFP positive expressing cells collected were separately reseeded to monitor cultures purity overtime. The BD FACSDiva version 8.0 software was used.

3.4. Resazurin metabolism

Cells were seeded at 1.0 x 10^5 (PC12 cells) or 2.0 x 10^5 cells/mL (U2OS cells) in 96-well plates. After 3 days (PC12 cells) or 24h (U2OS cells), cells were treated with the Hsp70 ATPase activity inhibitors YM-1 and YM-08 (Sigma-Aldrich), dissolved in DMSO, or solvent at the indicated concentrations. After 20h of treatment, 40 µM resazurin was added to the cells and its reduction to resorufin was assessed by fluorescence readings during 4h (24h of treatment). Readings were performed with a Synergy HT Plate Reader (BioTek), at 530nm excitation and 590nm emission.

3.5. Heat shock and drugs

Heat shock was performed by incubating cells for 1h in a pre-heated water bath at 42°C. Cells were left to recover at 37°C for 7h to allow protein synthesis. Cells were treated with 3µM YM-1 in 0.1% DMSO for 24h for all the following experiments.

3.6. Plasmids and antibodies

Plasmids: EGFP-Htt^{ex1}Q23 and EGFP-Htt^{ex1}Q74 (40261 and 40262, Addgene), RFP-β-actin was a gift from Pedro Guedes-Dias (University of Pennsylvania). Primary antibodies and dilutions for immunoblotting: anti-Hsp90 (sc-13119; 1:1000), anti-Hsp70 (sc-66049; 1:1000), anti-mtHsp70 (sc-133137; 1:1000), anti-ubiquitin (sc-8017; 1:1000) and anti-GFP (sc-9996; 1:1000) were from Santa Cruz Biotechnology; anti-Hsp70 (PA5-28003; 1:3000), anti-Hsp40 (PA5-17382; 1:1000) and anti-RFP (MA5-15257; 1:3000) were from Invitrogen; anti-Hsp110 (13383-1-AP; 1:2000 for western blot and 1:500 for filter trap immunoblot) was from Proteintech; anti-polyQ (MAB1574; 1:3000) was from Merck Millipore; anti-Htt 4-19 N-terminal (CH00146; 1:500) was from Coriell Institute.

3.7. Life cell imaging

3.7.1. U2OS cells

Cells were seeded at 1.5 x 10^5 cells/mL in 8-well glass-bottom µ-slides (Ibidi) and transfected with wild-type Htt (Q23) or mHtt (Q74) encoding plasmids, as described above. 16-18h post-transfection, cells were treated with YM-1 or solvent. After 24h of treatment,

cells were incubated with 1µg/ml Hoechst 34580 for 30 minutes, washed and live imaged in Hibernate medium supplemented with 10% fetal bovine serum at 37°C. For visualization of active mitochondria, cells were incubated with 10 nM $TMRM^+$ for 30 minutes at 37ºC prior to imaging. Fluorescently tagged wild-type Htt (EGFP-Httex1Q23) and mHtt (EGFP-Httex1Q74) were excited at 488 nm, Hoechst at 380 nm, and $TMRM^+$ at 557nm. EGFP positive cells were scored as diffuse, aggregated or dead (shrunken cells with condensed DNA). Images were acquired with an Eclipse TE300 microscope system (Polychrome II monochromator, TILL Photonics; ORCA-ER C4742-95 CCD camera, Hamamatsu; ProScan motorized stage, Prior; Micro-Manager 2.0 software, https://micro-manager.org). For fluorescence intensity comparisons, non-saturated images were acquired with identical equipment settings. Image analysis was performed with ImageJ (https://imagej.nih.gov.ij/).

3.7.2. PC12 cells

PC12 cells were seeded at 1.5 x 10^5 cells/mL in 35mm gridded glass-bottom µ-dishes (Ibidi). After 3 days, cells were induced to express Htt with 5µM Ponasterone A. For experiments with cells differentiation, 50ng/mL nerve growth factor (NGF) was used. Cell viability was assessed by incubating cells with 1µM rhodamine 123 for 15 minutes, followed by a wash prior to live imaging. Fluorescently tagged wild-type Htt (RFP-FL-HttQ23) and mHtt (RFP-FL-HttQ73 or RFP-FL-HttQ145) were excited at 557nm and rhodamine 123 at 500nm. Images were acquired with the Eclipse TE300 system and processed with ImageJ.

3.8. Immunofluorescence

For staining of active mitochondria, cells were incubated with 300nM MitoTracker Deep Red for 30 min at 37ºC and washed 3 times for 10 min with pre-warmed culture media, prior to the fixation. Cells were fixed with 4% paraformaldehyde for 15 min at 37°C, washed in PBS, and permeabilized and blocked with 0.1% Triton X-100 and 3% BSA in PBS (Abdil – antibody dilution buffer) for 30 min. Cells were then incubated with the primary antibody: anti-Hsp70 (1:200; PA5-28003, Invitrogen) or anti-Htt 4-19 N-term (1:200; CH00146, Coriell Institute), for 1h or overnight at 4°C in Abdil and washed with 0.1% Triton X-100 in PBS. After that, cells were incubated for 1h with the respective AlexaFluor-488 and -647 conjugated secondary antibodies (A-21245 or A-11034; 1:200, Invitrogen) in Abdil and washed in PBS. For DNA staining, cells were then incubated with 1µg/ml Hoechst 34580 for 5 min and washed in PBS. Coverslips were assembled in fluorescent mounting medium

(Dako) and cells were imaged by fluorescence microscopy with the Eclipse TE300 system. Image analysis was performed with ImageJ or CellProfiler (Broad Institute).

3.9. Western blot

Cells were seeded at 2.0 - 4.0 x 10^5 cells/mL in 6-well plates. At the indicated timepoints, cells were rinsed with ice-cold PBS and lysed in buffer containing 50mM Tris (pH 8.0), 1% NP-40, 1% sodium deoxycholate, 150mM NaCl, 1mM EDTA, 0.1% SDS and protease inhibitor cocktail (Thermo Scientific). After a soft homogenization with Precellys® lysis kit and 3 freeze-thaw cycles, the lysates were centrifuged at 600 x g, for 10 min at 4°C, and supernatant protein concentrations were quantified by Bradford's method. Protein samples were denatured (70°C, 10 min) in lithium dodecyl sulfate (LDS) loading buffer (Invitrogen), loaded (20-25µg protein) into polyacrylamide gels (3-8% Tris-acetate or 4-12% Bis-Tris NuPAGE precast gels), electrophoresed (150V, 50 min for 3-8% gel; 200V, 30 min for 4-12% gel), and transferred into PVDF membranes (20-25V, 8 min). Membranes were blocked with 5% bovine serum albumine (BSA) in PBS with 0.05% Tween 20 (PBST 0.05%), then incubated with primary antibodies, followed by washing with PBST 0.05% and incubation with the respective secondary antibodies conjugated with horseradish peroxidase for detection using Novex ECL Chemiluminescent kit (Invitrogen) and Chemidoc MP Imaging System (Bio-Rad). Membrane coomassie staining was used as loading control for induced cells and RFP-β-actin levels was used for data normalization of transfected cells. Densitometric analyses were performed with ImageJ.

3.10. Filter trap assay

Protein extraction and quantification was performed as described above. Protein samples (15-25µg) in 2% SDS buffer (2% SDS in PBS) were incubated 5 minutes at 95°C and filtered through a 0.2µm pore size cellulose acetate membrane (Sterlitech) assembled on a 0.45µm pore nitrocellulose membrane (GE Healthcare) pre-equilibrated with PBS buffer (Bio-Dot Microfiltration Apparatus, Bio-Rad). For comparisons with proteins in their native state, protein samples diluted in PBS in absence of denaturing conditions were also used. Membranes were blocked with 5% BSA in PBST 0.05% for further aggregates detection by immunoblot. Nitrocellulose membrane coomassie staining was used as loading control.

3.11. Immunoprecipitation

Protein extraction and quantification was performed as described above. For immunoprecipitation SureBeads ™ Protein G Magnetic Beads (Bio-Rad) were used. Briefly, 1mg beads containing 9-12µg protein G were washed 3 times with PBST 0.1% prior to incubation with anti-Hsp70 antibody (1:500; PA5-28003, Invitrogen) for 10 min. After incubation, beads were washed 3 times with PBST 0.1% to remove unbound antibody and then incubated with 500µg of protein sample for 1h. After 3 washes with PBST 0.1% to remove unbound protein fractions, bound proteins were eluted in LDS buffer with incubation for 10 min at 70°C. Supernatant was loaded into polyacrylamide gels and analyzed by immunoblotting.

3.12. Statistical analysis

Values are mean ± standard error of the mean of the *n* specified in figure legends. For normally distributed data, t-test or one-way ANOVA with Sidak post-Hoc were used to compare two or more groups, respectively. The Kruskal-Wallis ANOVA with Dunn's post-Hoc was used for non-normally distributed data. Two- or three-way ANOVA with Sidak's post-Hoc were used to test two or three factors, respectively, and their interaction (genotype x treatment; heat shock x treatment; genotype x heat shock x treatment). Time-dependent curves were fitted with non-linear regression. Differences between two curves were tested with the sum-of-squares F test. Data analysis were performed with Prism 6.0 (GraphPad software) or SPSS Statistics (IBM). Differences were considered statistically significant when $P < 0.05$.

CHAPTER IV

RESULTS

4. Results

4.1. Characterization of the PC12 cell line expressing inducible full-length huntingtin

Initially, for use as an HD model of full-length Htt, we obtained from CHDI Foundation (Coriell Institute for Medical Research) a PC12 cell line with inducible expression of wild-type or mHtt with 23 or 73 glutamines with an RFP tag (FL-Htt-Q23 and FL-Htt-Q73), respectively. Induced cells were live imaged to confirm the expression of RFP. In the presence of ponasterone A, both lines exhibited the expression of RFP (Figure 4.1A).

In an attempt to work with a neuronal-like cellular model, we tested differentiation of FL-Htt-Q73 expressing cells with nerve growth factor (NGF). In response to NGF, PC12 cells differentiate, exhibiting a typical phenotype of neuronal cell, extending neurites (Greene and Tischler, 1976). Indeed, in our model, a small fraction of the NGF treated cells exhibited morphological changes characterized by the appearance of neurite-like extensions starting from 24h of treatment (Figure 4.1B). However, preliminary data suggested that cell differentiation induced a decrease in Htt levels and viability in FL-Htt-Q73 cells compared to undifferentiated cells (Figure 4.1C and D).

While all the cells from the FL-Htt-Q23 line presented RFP fluorescence, only a fraction of the FL-Htt-Q73 cells expressed RFP. Thus, in order to specifically isolate the RFP expressing cells from the FL-Htt-Q73 culture, we performed two-cycles of fluorescence activated cell sorting (FACS) (Figure 4.1E). Both the RFP negative and RFP positive populations collected were separately reseeded. Overtime monitorization of the RFP positive cells collected from sorting revealed that these cells did not maintained the expression of the transgene. For that reason, we considered the replacement of this line for one expressing the full-length mHtt with 145 glutamines. In contrast to FL-Htt-Q73 cells, and despite the low RFP intensity levels, all FL-Htt-Q145 cells expressed RFP when induced (Figure 4.1A). We thus proceed our studies of full-length Htt proteostasis using FL-Htt-Q23 and FL-Htt-Q145 cell lines.

Preliminary *in situ* immunofluorescence experiments suggest that there is no aggregation of mHtt in this cell model, as both FL-Htt-Q23 and FL-Htt-Q145 expressing cells presented a diffuse distribution of Htt when labeled with an N-terminal Htt antibody (Figure 4.1F). To characterize the proteostasis of full-length Htt the levels of FL-Htt-Q23 and FL-Htt-Q145 were monitored overtime by western blot. PC12 cells exhibited a non-significant time-dependent increase in both FL-Htt-Q23 and FL-Htt-Q145 levels in the presence of ponasterone A (Figure 4.2C). The turn off of transgene expression by removal of ponasterone A from the culture media after 3 days induction, for 1 days, resulted in a

decrease in both FL-Htt-Q23 and FL-Htt-Q145 levels, however, this decrease was more pronounced in mHtt expressing cells, which suggests a faster degradation kinetics of mHtt compared with wild-type Htt (Figure 4.2C).

4.2. Full-length mutant huntingtin is a client protein of the molecular chaperone Hsp70

To investigate if the expression of full-length mHtt activates an HSR in these cells, the levels of different Hsp members were measured overtime. The expression of either FL-Htt-Q23 or FL-Htt-Q145 did not alter the levels of Hsp70 or other stress-induced cytosolic chaperones, namely Hsp90 and Hsp40, neither at 1 or 4 days induction, compared to uninduced cells (Figure 4.2D), indicating that the expression of full-length mHtt does not activate an HSR in PC12 cells. Moreover, the levels of the mitochondrial Hsp70, mtHsp70, were also unchanged by the expression of FL-Htt-Q23 or FL-Htt-Q145, compared to uninduced cells, suggesting that the expression of full-length mHtt does not activate a mtUPR (Figure 5.2D). To understand if the chaperone machinery plays a role in full-length Htt proteostasis, we assessed Hsp70 interaction with full-length Htt. Immunoprecipitation with an anti-Hsp70 antibody analysis showed that Hsp70 interacts with FL-Htt-Q145 but not FL-Htt-Q23 (Figure 4.2E), indicating a specific recognition of the misfolded protein by the chaperone machinery.

Collectively, these results indicate that the chaperone machinery plays a role in full-length mHtt proteostasis in our model. They also show that the expression of full-length mHtt does not elicit an activation of the HSR to increase chaperone levels, thus limiting chaperone availability to deal with mHtt accumulation.

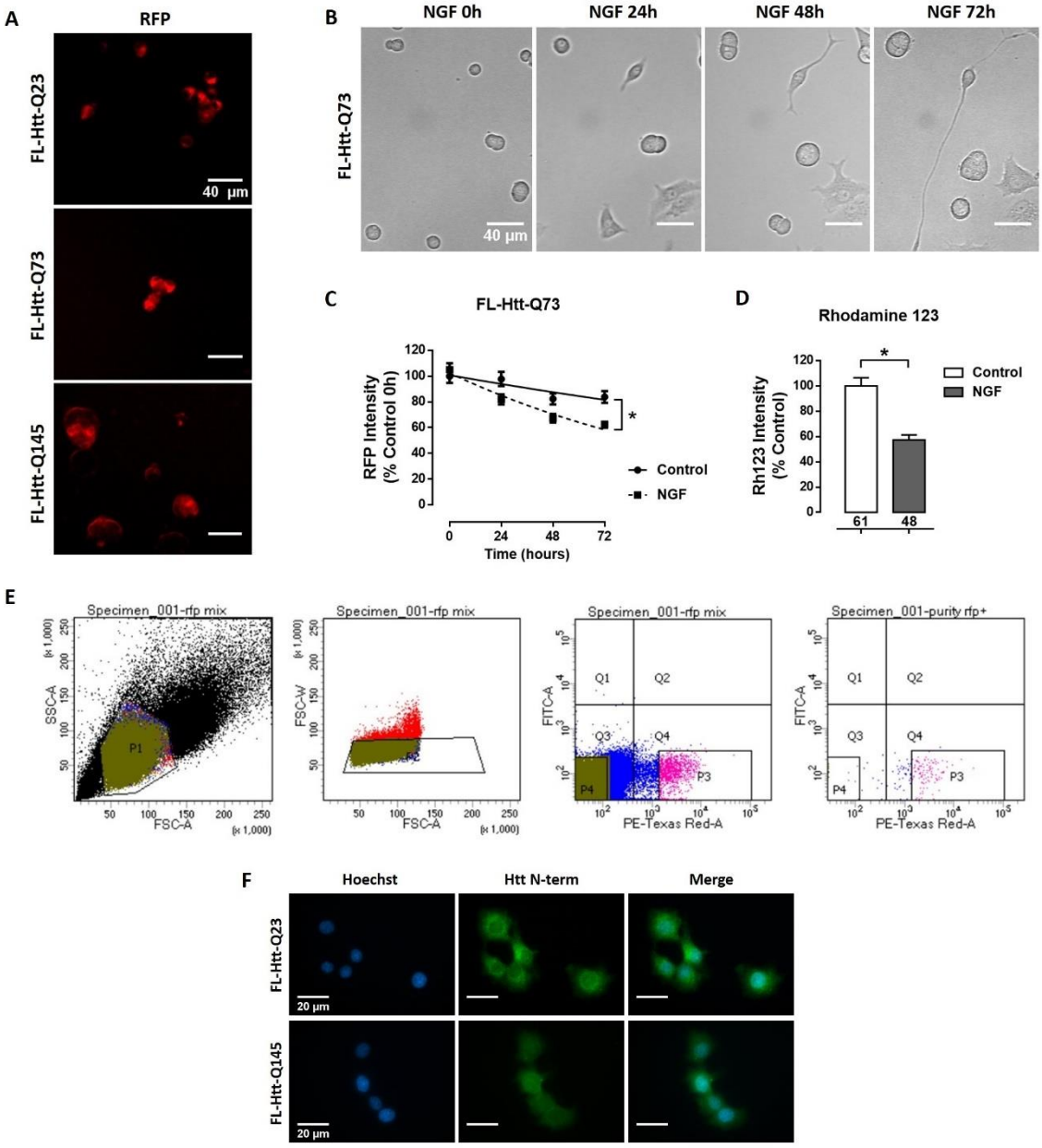

Figure 4.1. PC12 cell model. (A) Representative fluorescence microscopy images of Htt expression in PC12 cells inducibly expressing FL-Htt-Q23 and FL-Htt-Q73 for 8 days, and FL-Htt-Q145 for 5 days. (B) Representative images of FL-Htt-Q73 expressing cells differentiation with NGF. (C) Quantification of RFP intensity in FL-Htt-Q73 expressing cells; n=111 (Control) and 136 cells (NGF), **P*<0.05, non-linear regression with sum-of-squares *F* test. (D) Quantification rhodamine 123 intensity; n=48-61 cells, **P*<0.05, unpaired *t*-test. (E) FACS analysis of PC12 cells inducibly expressing FL-Htt-Q73 for 48h. Positive FL-Htt-Q73 cells were sorted based on their granularity/complexity (SSC – side-scattered), size (FSC – forward-scattered) and red fluorescence. (F) Representative immunofluorescence images of PC12 cells induced for 4 days showing nucleus staining (Hoechst) and Htt expression (N-terminal Htt antibody).

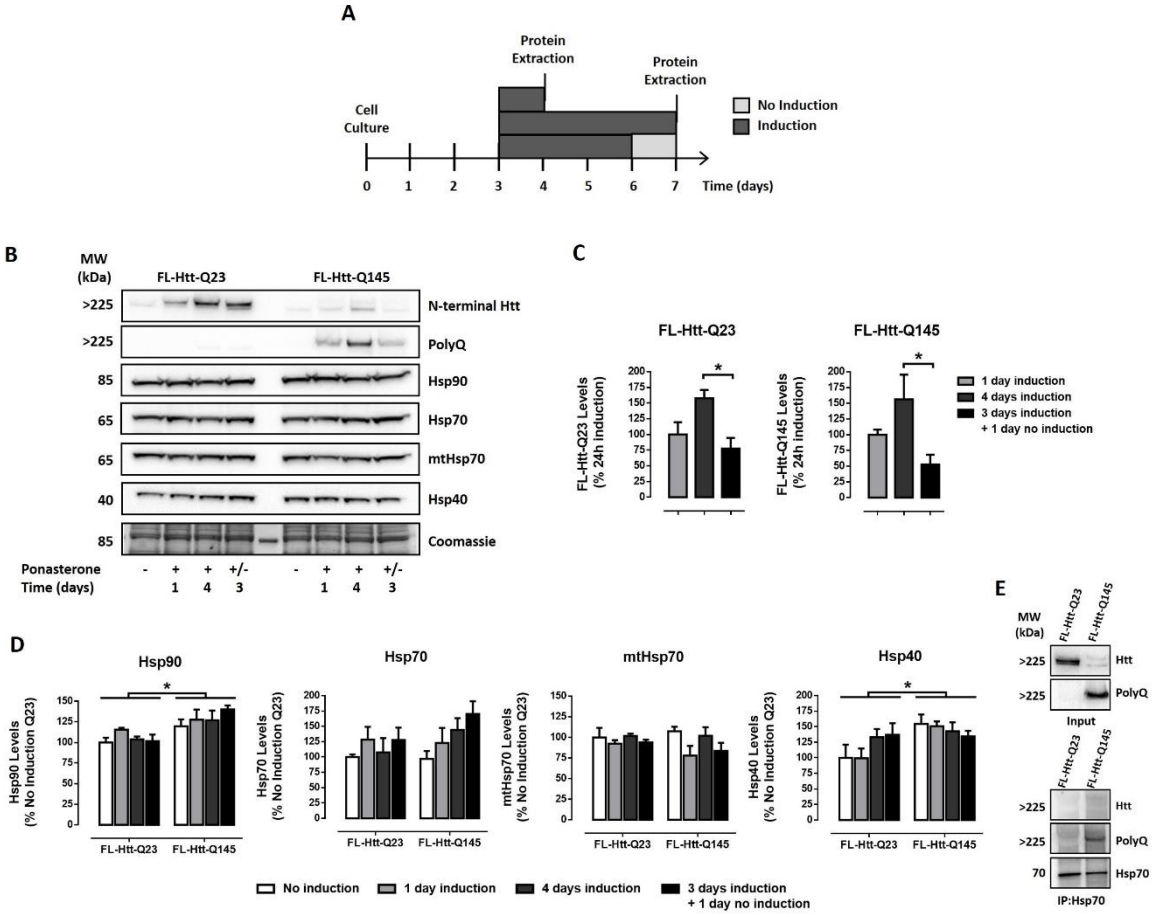

Figure 4.2. Full-length huntingtin and heat shock proteins expression in PC12 cells. (A) Schematic experimental design. PC12 cells were incubated with 5µM Ponasterone A for different times to induce the expression of full-length Htt. To turn off transgene expression, Ponasterone A was removed from the culture media after 3 days induction, for 1 day. The detection of wild-type Htt was performed with an anti-Htt 4-19 N-terminal antibody (1:500; rabbit; CH00146) and the detection of mutant Htt was performed with an anti-polyQ antibody (1:3000; mouse; MAB1574). (B) Representative Western blots for the levels of full-length Htt and different Hsp. (C) Quantification of full-length Htt levels; n=4 independent experiments, **P*<0.05, one-way ANOVA with Sidak post-Hoc. (D) Quantification of Hsp levels; n=4 independent experiments, **P*<0.05, two-way ANOVA. (E) Representative immunoblot of immunoprecipitation with antibody anti-Hsp70 in cells induced for 4 days.

4.3. Hsp70 modulation decreases full-length mutant huntingtin levels in PC12 cells

For the following experiments with PC12 cells the induction time used was 4 days to ensure high expression levels of both FL-Htt-Q23 and FL-Htt-Q145. Exposing cells to heat shock (HS) conditions (42°C; 1h) alone only slightly increased the expression of Hsp70 in both FL-Htt-Q23 and FL-Htt-Q145 expressing cells (Figure 4.3.2E). Interestingly, the levels of the Hsp70 co-chaperone, Hsp40, were only increased by HS in cells expressing FL-Htt-Q145 (Figure 4.3.2E), suggesting a more relevant role of this chaperone in mHtt expressing cells compared to wild-type cells. Collectively, these results on the effect of HS indicate that the HSR is not impaired in cells expressing full-length mHtt.

As the expression of full-length mHtt was not able to activate an HSR in order to increase chaperone availability to cope with mHtt accumulation, we wondered if the induction of the HSR would be enough to alter full-length mHtt proteostasis. We observed that the HS alone did not alter the levels of either FL-Htt-Q23 or FL-Htt-Q145, compared to control conditions (Figure 4.3.2C). We also investigated whether pharmacological modulation of Hsp70 with ATPase inhibitors alone or in conditions of HSR induction alter Htt proteostasis.

To determine the concentrations of the Hsp70 ATPase inhibitors to be used, we used resazurin metabolism as an indicator of cell viability. Resazurin is a cell permeable weakly fluorescent blue dye that can be irreversibly reduced to the highly fluorescent pink resorufin by metabolically active cells (Czekanska, 2011). Different concentrations of the Hsp70 ATPase activity inhibitor YM-1 (0.3-30µM) and its neutral analog YM-8 (0.3-10µM) were tested (Figure 4.3.1A). Solubility issues with YM-8 dictated the exclusion of this drug from the study, as YM-8 solutions starting from the concentration 3µM exhibited precipitates (Figure 4.3.1E). The concentration of 3µM YM-1 was selected after resazurin metabolism assays in both PC12 (non-induced) and U2OS (non-transfected) cells as the highest concentration that induced a decrease in metabolism under 20% in both cell lines, being within the range of concentrations used for this molecule in the literature (Figure 4.3.1B and C).

Treatment with YM-1 alone did not alter the levels of either FL-Htt-Q23 or FL-Htt-Q145 (Figure 4.3.2C). It also did not affect the levels of Hsp70 or its co-chaperone Hsp40 (Figure 4.3.1E). However, the combination of YM-1 with HS induced a decrease in FL-Htt-Q145 (53%) but not FL-Htt-Q23 levels, compared to controls (Figure 4.3.2C). Since HS increased the levels of Hsp70 and its co-chaperone Hsp40 in FL-Htt-Q145 expressing cells treated with YM-1, these results support an Hsp70-dependent effect of YM-1 (Figure 4.3.2E). Concerning other chaperones, the levels of Hsp90 were only affected by YM-1 in

cells expressing FL-Htt-Q145 (Figure 4.3.2E). In these cells, treatment with YM-1 decreased the levels of Hsp90, a chaperone that is thought to mediate mHtt accumulation through the recruitment of the deubiquitinase Usp19, which deubiquitinates mHtt, decreasing its degradation (He et al., 2016; He et al., 2017). The levels of the mitochondrial Hsp70, mtHsp70, were also only affected by YM-1 in cells expressing FL-Htt-Q145 (Figure 4.3.2E). In this case, treatment with YM-1 increased the levels of mtHsp70, whose functions are mitochondrial protein import and subsequent folding (Chacinska et al., 2009; Schmidt et al., 2010).

Since the UPS is compromised in HD (Bennett et al., 2007) and Hsp70 is able to redirect misfolded proteins for UPS mediated degradation (Pratt et al., 2010), we also assessed the levels of ubiquitinated proteins in our model as an indicator of the global state of this degradation pathway. The expression of FL-Htt-Q145 did not alter the overall levels of ubiquitinated proteins compared to FL-Htt-Q23 (Figure 4.3.2D), indicating that in our full-length mHtt model there is no impairment of the UPS. Neither exposing cells to HS or treating with YM-1 alone or in combination affected the levels of ubiquitinated proteins (Figure 4.3.2D).

Altogether, these results indicate that YM-1 alters full-length Htt proteostasis in an Hsp70-dependent manner, reducing the levels of mHtt without altering the levels of wild-type Htt. Moreover, YM-1 exerted this effect without changing the global levels of ubiquitinated proteins.

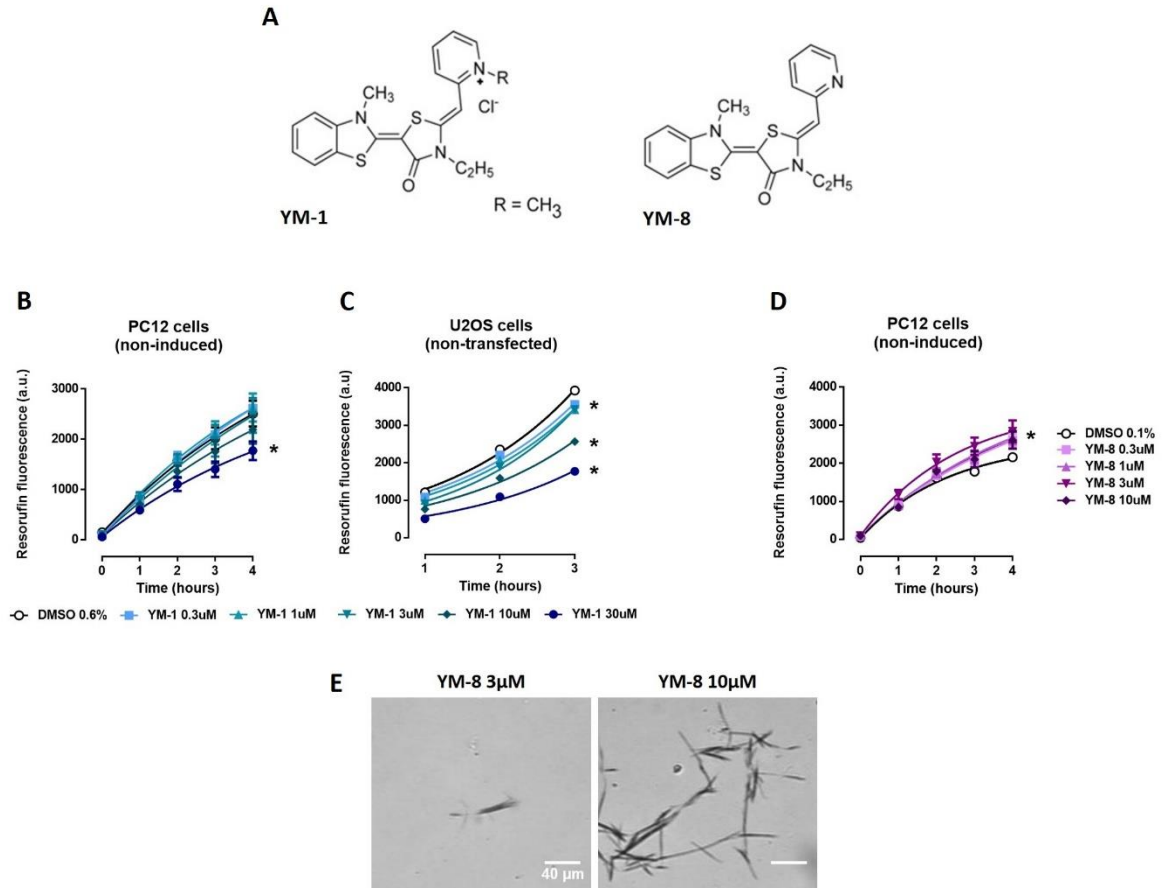

Figure 4.3.1. Effect of YM-1 and YM-8 on cell metabolism. (A) Chemical structure of YM-1 and YM-8. Quantification of resazurin metabolism in non-induced PC12 (B) and non-transfected U2OS cells (C) treated with different concentrations of YM-1; n=1 experiment in triplicates, **P*<0.05 vs DMSO, non-linear regression with sum-of-squares *F* test. (D) Quantification of resazurin metabolism in non-induced PC12 cells, treated with different concentrations of YM-8, n=1 experiment in triplicates, **P*<0.05 vs DMSO, non-linear regression with sum-of-squares *F* test. (E) Representative images of YM-8 precipitates.

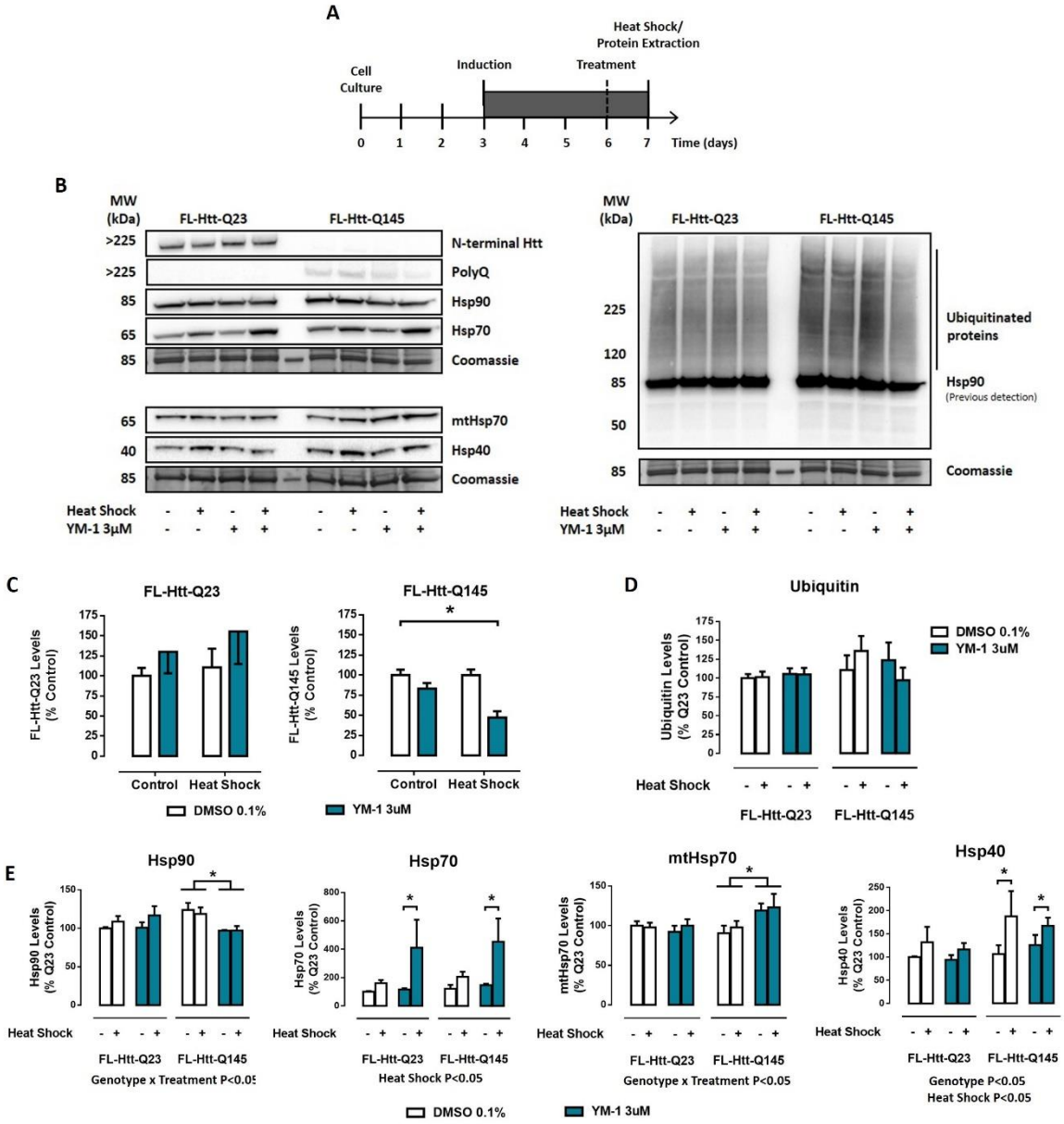

Figure 4.3.2. Effects of heat shock and YM-1 on full-length huntingtin proteostasis and the molecular chaperone machinery in PC12 cells. (A) Schematic experimental design. PC12 cells were incubated with 5µM Ponasterone A for 4 days to induce the expression of full-length Htt. Cells were treated with solvent or 3µM YM-1 for the last 24h of incubation and heat shock (42°C, 1h) was performed 7h prior to protein extraction. (B) Representative Western blots for the levels of full-length Htt, different Hsp and ubiquitinated proteins. The detection of wild-type Htt was performed with an anti-Htt 4-19 N-terminal antibody (1:500; rabbit; CH00146) and the detection of mutant Htt was performed with an anti-polyQ antibody (1:3000; mouse; MAB1574). (C) Quantification of full-length Htt levels; n=3-7 independent experiments, **P*<0.05, two-way ANOVA with Sidak post-Hoc. (D) Quantification of ubiquitinated proteins and (E) Hsp levels; n=3-5 independent experiments,

*P<0.05, three-way ANOVA with Sidak post-Hoc. Coomassie staining was used for loading control.

4.4. Hsp70 modulation decreases soluble N-terminal mutant huntingtin levels in U2OS cells

We then extended or study to an HD cellular model of N-terminal Htt in order to understand if different Htt species differentially affect the chaperone machinery. U2OS cells were transfected with an EGFP-exon1-Htt-Q23 or EGFP-exon1-HttQ74 construct alone or together with an RFP-β-actin construct and Htt and Hsp expression was monitored by western blot or fluorescence microscopy. Cells expressing wild-type Htt (NT-Htt-Q23) exhibit a diffuse Htt distribution, while cells expressing mHtt (NT-Htt-Q74) exhibit both a diffuse and aggregated Htt distribution. These aggregates can be present in both the nucleus and the cytosol, being more frequently found in the nucleus (89% of the cells expressing mHtt aggregates exhibit nuclear aggregates whereas only 32% exhibit cytosolic aggregates) (Figure 4.4B).

The assessment of Hsp levels by western blot showed that the expression of NT-Htt-Q74 did not alter the levels of Hsp70 or the other stress-induced cytosolic chaperones evaluated, compared to NT-Htt-Q23 expressing cells (Figure 4.4F), indicating that, as full-length mHtt in PC12 cells, the expression of N-terminal mHtt does not activate an HSR in this model. Similarly, the levels of mtHsp70 were unchanged by the expression of N-terminal mHtt (Figure 4.4F). Regarding the levels of ubiquitinated proteins, these were also unchanged in cells expressing NT-Htt-Q74 compared to NT-Htt-Q23 cells, suggesting an identical activity of the UPS between the two genotypes. Treatment with YM-1 alone significantly decreased the levels of soluble NT-Htt-Q74 (27%) without altering the levels of soluble NT-Htt-Q23 (Figure 4.4D). Moreover, YM-1 exerted this effect without affecting the levels of either the ubiquitinated proteins or the assessed stress-induced cytosolic Hsp in comparison with solvent treated cells (Figure 4.4E and F). However, treatment with YM-1 increased the expression of mtHsp70 in both NT-Htt-Q23 and NT-Htt-Q74 expressing cells, suggesting an activation of mtUPR (Figure 4.4F).

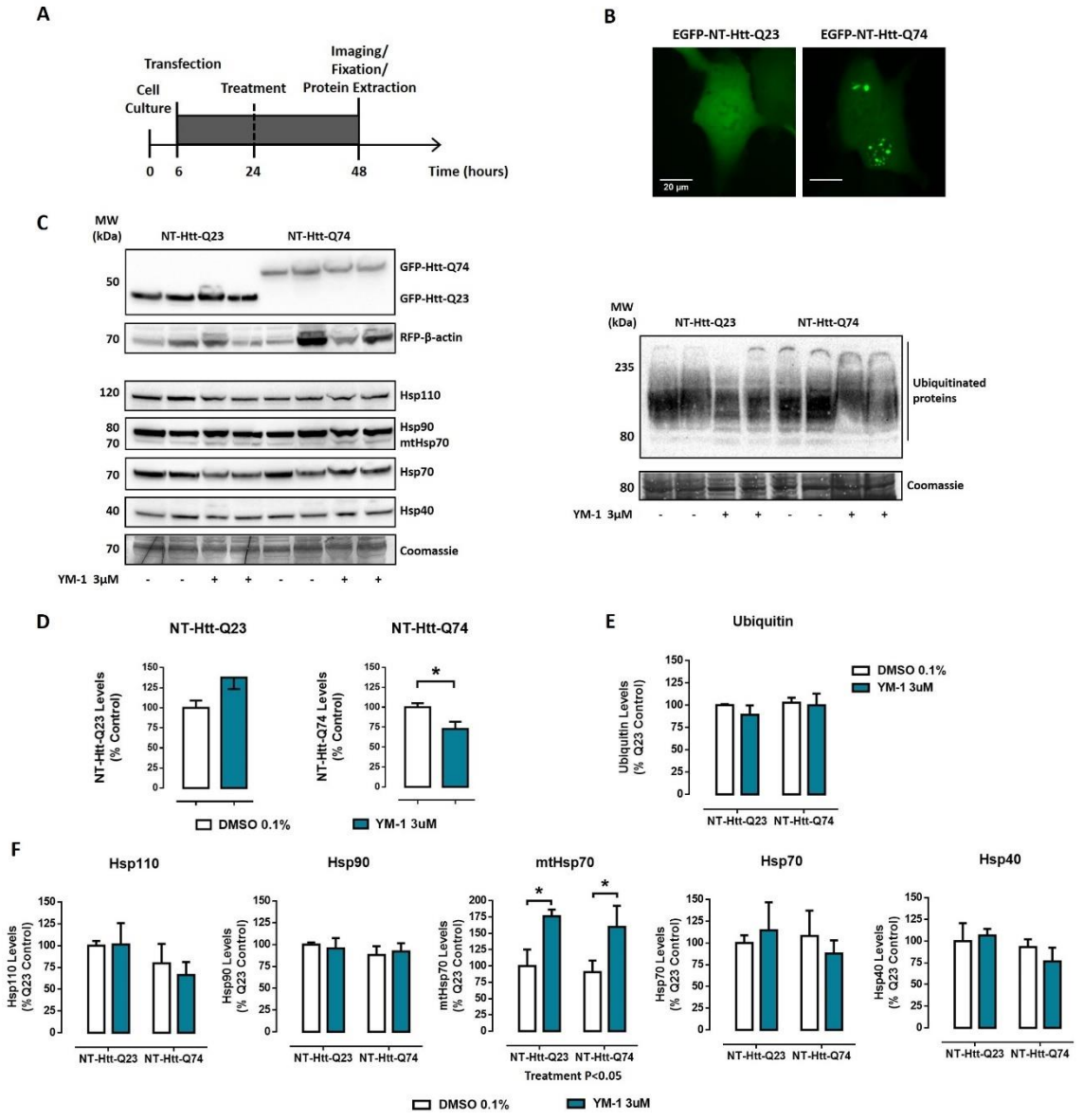

Figure 4.4. Effects of YM-1 on N-terminal huntingtin proteostasis and the molecular chaperone machinery in U2OS cells. (A) Schematic experimental design. U2OS cells were transfected with EGFP-NT-Htt-Q23 or Htt-Q74 alone (for imaging experiments) or co-transfected with EGFP-NT-Htt-Q23 or Htt-Q74 and RFP-β-actin (for immunoblot experiments). 16-18h post-transfection, cells were treated with solvent or 3µM YM-1 for 24h. (B) Representative fluorescence microscopy images of Htt expression in NT-Htt-Q23 and NT-Htt-Q74 expressing cells. (C) Representative Western blots for the levels of N-terminal Htt, different Hsp and ubiquitinated proteins. (D) Quantification of N-terminal Htt levels; n=3 independent experiments, **P*<0.05, paired *t*-test. Levels of RFP-β-actin were used for data normalization. (E) Quantification of ubiquitinated proteins and (F) Hsp levels; n=3 independent experiments, **P*<0.05, two-way ANOVA. Coomassie staining was used for loading control.

4.5. N-terminal mutant huntingtin aggregation is unaffected by Hsp70 modulation in U2OS cells

The assessment of Hsp70 distribution by in situ immunofluorescence showed that Hsp70 accumulated around NT-Htt-Q74 aggregates, suggesting that Hsp70 may also play a role in N-terminal mHtt aggregation and justifying the evaluation of YM-1 effects on mHtt aggregation (Figure 4.5A). Indeed, YM-1 was shown to decrease the levels of aggregated mutant AR in the cellular model of SBMA (Wang et al., 2013). In order to investigate whether treatment with YM-1 alters N-terminal mHtt aggregation, cells were live imaged and live EGFP positive cells were divided as having a diffuse or aggregated Htt profile. In contrast to the results observed in the SBMA model, treatment with YM-1 increased the proportion of cells with N-terminal mHtt aggregates (Figure 4.5B). To understand if this increase in the proportion of cells with aggregates is related with an increase in SDS-resistant mHtt aggregates, we next performed filter trap assays for the isolation and detection of SDS-resistant aggregates by immunoblot. Cells expressing NT-Htt-Q74 exhibited SDS-resistant Htt aggregates which were not detected in NT-Htt-Q23 expressing cells (Figure 4.5D). Treatment with YM-1 did not alter the levels of SDS-resistant mHtt aggregates, compared to control (Figure 4.5E).

YM-1 effect on the levels of aggregated mutant AR was attributed to an increase in protein degradation by the proteasome as proteasomal inhibition with MG-132 blocked the decrease of mutant AR aggregation induced by YM-1 (Wang et al., 2013). Suppression of protein aggregation can be explained by an increase in soluble protein degradation through the proteasome, however, the proteasome-mediated degradation of already formed protein aggregates would require a previous disaggregase activity. Thus, we next asked if N-terminal mHtt aggregation triggered the formation of the trimeric disaggregase complex constituted by Hsp70, Hsp40 and Hsp110. For that, we assessed the presence of these three chaperones in the SDS-resistant aggregates fraction. Immunoblot analysis revealed that, in contrast with Hsp70 which was always detectable, Hsp40 and Hsp110 were not always detected in SDS-resistant aggregates from both solvent and YM-1 treated cells (Figure 4.5D), suggesting that the expression of N-terminal mHtt does not trigger the formation of the disaggregation complex. Quantification of the Hsp70 levels in the SDS-resistant aggregates fraction showed that these were unchanged by YM-1 treatment (Figure 4.5E).

Altogether, results on the N-terminal Htt model showed that modulation of Hsp70 with YM-1 alters N-terminal Htt proteostasis, reducing the soluble levels of mHtt without altering mHtt aggregation. Moreover, treatment with YM-1 did not alter mHtt induced cell death evaluated by the proportion of dead cells trough live imaging (Figure 4.5C).

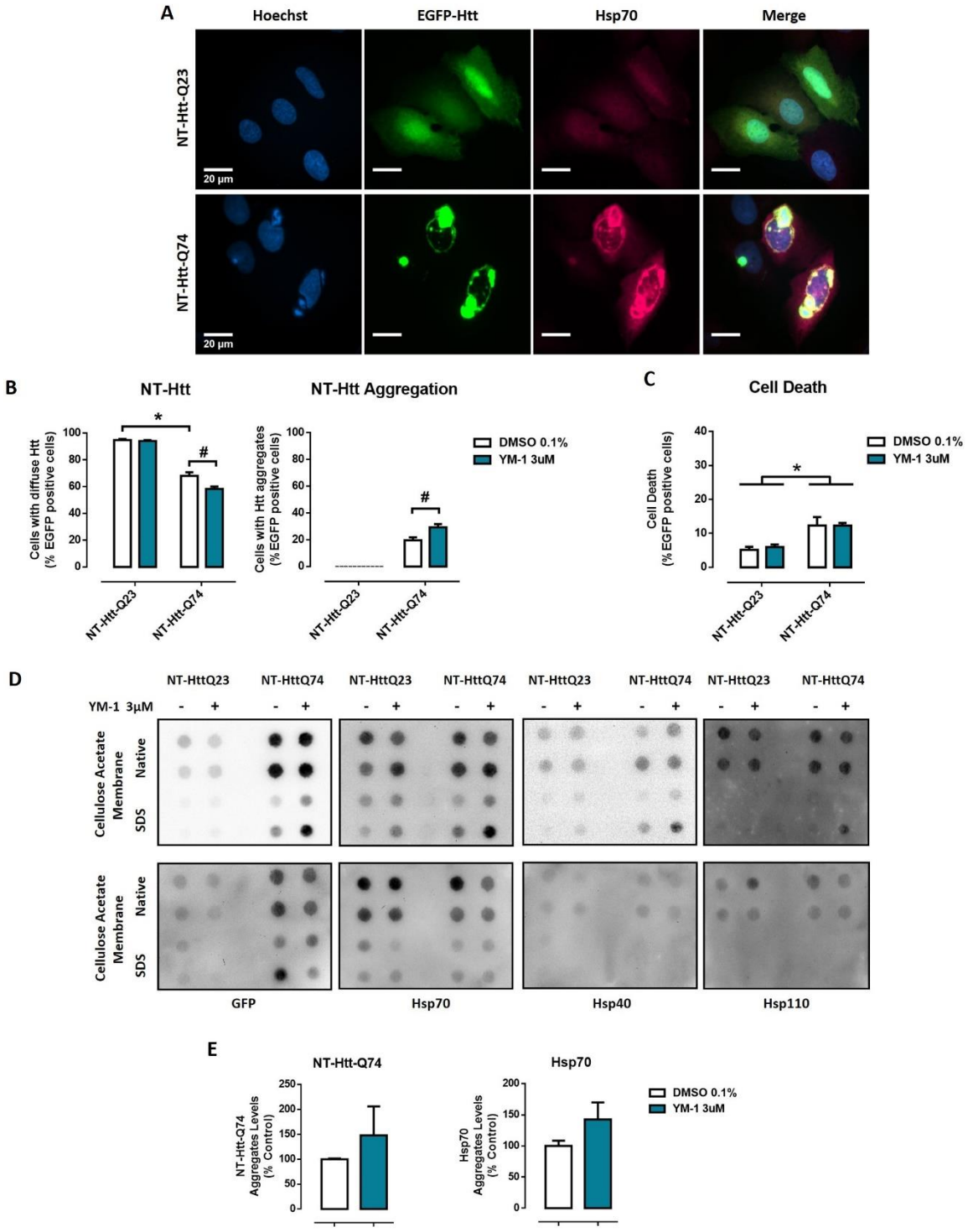

Figure 4.5. Effects of YM-1 on N-terminal huntingtin aggregation and viability in U2OS cells. Experimental design as in Figure 3. (A) Representative immunofluorescence images of NT-Htt-Q23 or NT-Htt-Q74 expressing cells showing nucleus staining (Hoechst), Htt (EGFP) and Hsp70 expression (AlexaFluor 647). (B) Quantification of the proportion of cells with diffuse or aggregated N-terminal Htt. Cells were incubated with Hoechst, washed and live imaged. EGFP positive cells were scored as live or dead and live cells were further

divided as having a diffuse or aggregated Htt profile, n=6 independent experiments, **P*<0.05 vs HttQ23 control, #*P*<0.05 vs control, two-way ANOVA and paired *t*-test. (C) Quantification of the proportion of death cells; n=6 independent experiments, **P*<0.05, two-way ANOVA. (D) Representative immunoblots of filter trap assay of N-terminal Htt SDS-resistant aggregates. Protein lysates diluted in PBS or 2% SDS were filtered through a 0.2µm pore size cellulose acetate membrane assembled on a 0.45µm pore nitrocellulose membrane. Protein extracts from the same sample were applied in different membranes probed with different antibodies. The upper and the lower panel represent 2 independent experiments, respectively. (E) Quantification of N-terminal Htt and Hsp70 levels in SDS-resistant aggregates fraction; n=3 independent experiments, paired *t*-test. Nitrocellulose membrane coomassie staining was used for loading control.

4.6. Hsp70 modulation prevents MitoTracker Deep Red intensity decrease induced by the expression of N-terminal mutant huntingtin

Mitochondrial dysfunction may play a role in mHtt proteostasis through a decrease in ATP production, which is required to sustain the ATP-dependent activities of the proteostasis network (Hutt and Balch, 2013). YM-1 is fluorescent at the same wavelength that EGFP, allowing its visualization through microcopy. Live imaging experiments revealed co-localization of YM-1 with $TMRM^{+}$-stained mitochondria, indicating that YM-1 can accumulate in the mitochondria and supporting the study of YM-1 effects on mitochondrial function (Figure 4.6A).

Preliminary experiments suggest that NT-Htt-Q74 expression decreases MitoTracker Deep Red intensity compared to NT-Htt-Q23 (Figure 4.6C). This decrease was prevented by YM-1 treatment (Figure 4.6C). MitoTracker Deep Red has been used as a mitochondrial potential dependent dye as its accumulation changes along with mitochondrial membrane potential (Greene et al., 2012; Mot et al., 2016; Xiao et al., 2016). Thus, these results suggest that the expression of mHtt is decreasing mitochondrial membrane potential and treatment with YM-1 is preventing this decrease.

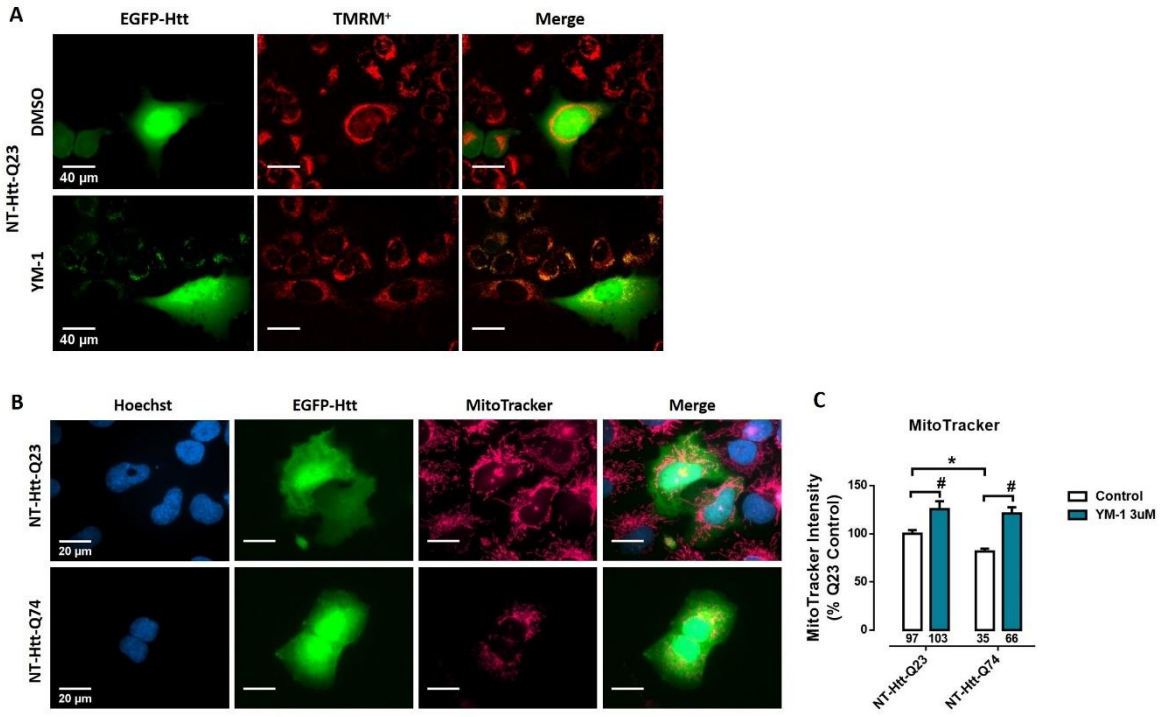

Figure 4.6. Effects of YM-1 on mitochondrial membrane potential in U2OS cells expressing N-terminal huntingtin. Experimental design as in Figure 3. (A) Representative fluorescence microscopy images of NT-Htt-Q23 expressing cells treated with solvent or 3µM YM-1. Cells were incubated with 10nM $TMRM^+$ and live imaged. (B) Representative fluorescence microscopy images of fixed NT-Htt-Q23 or NT-Htt-Q74 expressing cells showing nucleus (Hoechst) and mitochondria (300nM MitoTracker Deep Red) staining. (C) Quantification of MitoTracker intensity; n=35-103 cells per condition, $^*P<0.05$ vs HttQ23 control, $^{\#}P<0.05$ vs control, Kruskal-Wallis ANOVA with Sidak's post-Hoc.

CHAPTER V

DISCUSSION AND CONCLUSION

5. Discussion

5.1. Molecular chaperone machinery and heat shock response in full-length huntingtin expressing cells

In our work, for the study of full-length Htt proteostasis we used PC12 cell lines with inducible expression of full-length Htt with 23 or 145 glutamines (CHDI Foundation) as cells expressing full-length Htt with 73 glutamines were observed to not maintain the expression of the transgene. To our knowledge, Htt proteostasis in this particularly inducible cell model has not been characterized yet. For that reason, we monitored the levels of FL-Htt-Q23 and FL-Htt-Q145 overtime and after the turn off of transgene expression. The turn off of transgene expression resulted in a more pronounced decrease in FL-Htt-Q145 than FL-Htt-Q23 levels, suggesting a faster degradation kinetics of mHtt. This result is in accordance with previous studies showing that the mean lifetime of soluble mHtt is shorter than wild-type Htt in primary cultures of neurons (Tsvetkov et al., 2013). These results are consistent with a selective recognition and targeting of mHtt for accelerated degradation. More recently, a study on Htt degradation in different subcellular compartments, reported that soluble mHtt is degraded faster than wild-type Htt in the cell body of neurons but not in the neurites, where mHtt degradation was slower than wild-type Htt, thus suggesting a compartment-dependent degradation of mHtt (Zhao et al., 2016).

The levels of different Hsp are reduced or unaltered in models of HD, which indicates a possible impairment of the HSR (Yamanaka et al., 2008; Chafekar and Duennwald, 2012; Neueder et al., 2017; Scior et al., 2018). To understand the interplay of the molecular chaperone machinery and huntingtin proteostasis we measured the levels of different Hsp overtime and assessed Hsp70 interaction with full-length Htt in cells expressing FL-Htt-Q23 or FL-Htt-Q145. The results of the immunoprecipitation support the previous hypothesis of a selective targeting of mHtt for accelerated degradation as they showed an interaction of Hsp70 with FL-Htt-Q145 but not FL-Htt-Q23, indicating a selective recognition of mHtt by the chaperone machinery. Compared to uninduced cells, the expression of either FL-Htt-Q23 or FL-Htt-Q145 did not alter the levels of the stress-induced Hsp70, suggesting that the expression of full-length mHtt does not activate an HSR. Nevertheless, when we exposed cells to HS, the levels of Hsp40 increased in FL-Htt-Q145 expressing cells, indicating that the HSR is not impaired in these cells. These results are in line with previous studies supporting the failure of the HSR as a progressive and tissue/region-dependent process. The levels of different Hsp, including Hsp70, were observed to be progressively decreased in cortex but increased in muscles of R6/2 mice (Neueder et al., 2017). Moreover, while, at an early stage disease, pharmacological

induction of the HSR with HSP990, an Hsp90 inhibitor, induced the expression of Hsp in R6/2 brain similarly to wild-type mice, this upregulation in R6/2 mice was compromised with disease progression (Labbadia et al., 2011). The increase in Hsp40 levels induced by HS observed exclusively in cells expressing FL-Htt-Q145, point to a more relevant role of this chaperone in mHtt expressing cells compared to wild-type cells. Indeed, Hsp40 overexpression alone or in combination with Hsp70 was protective in different HD models (Reis et al., 2017). Hsp40 plays a pivot role in Hsp70 activity, regulating the nucleotide state of the Hsp70 ATPase domain and recruiting Hsp70 to client proteins (Saibil, 2013), making Hsp40 a limiting factor for Hsp70 activity. Thus, under conditions of misfolded proteins accumulation, such as mHtt, Hsp40 is required for the molecular chaperone machinery to efficiently deal with misfolded proteins.

Despite the absence of HSR activation by the expression of full-length mHtt, the selective interaction of Hsp70 with FL-Htt-Q145 suggests that the chaperone machinery is trying to cope with mHtt accumulation and supports Hsp70 modulation as a promising strategy to alter Htt proteostasis in this model.

5.2. Effects of Hsp70 modulation in full-length huntingtin proteostasis

As the HS alone had no effect on the levels of either FL-Htt-Q23 or FL-Htt-Q145 compared to control conditions, we investigated the potential of Hsp70 pharmacological modulation with YM-1 in full-length Htt proteostasis. YM-1 and its blood-brain permeable neutral analog, YM-8, are inhibitors of the Hsp70 ATPase activity which stabilize Hsp70 in its ADP-bound state, enhancing Hsp70 binding to client proteins (Miyata et al., 2013; Wang et al., 2013). In the polyQ disorder SBMA, treatment with YM-1 decreased the levels of both soluble and aggregated mutant AR in cells and ameliorated mutant AR induced toxicity in Drosophila (Wang et al., 2013). YM-1 and YM-8 were found to decrease the levels of tau, a protein known to misfold and accumulate in Alzheimer's disease, in brain tissues from tau transgenic mice (Abisambra et al., 2013; Miyata et al., 2013; Young et al., 2016). Preliminary resazurin metabolism assays revealed that YM-8 precipitates at the working concentrations tested, making impossible the assessment of its real effect on cells and explaining why none of the concentrations tested decreased the metabolism compared to the solvent (Figure 4.3.1D and E). For that reason, YM-8 was excluded from the study and we proceed with the experiments only with YM-1.

In contrast with the studies described above, in which YM-1 treatment was observed to decrease the levels of misfolded proteins, treatment with YM-1 alone did not alter the levels of either FL-Htt-Q23 or FL-Htt-Q145. However, combining YM-1 treatment

with HS conditions induced a decrease in the levels of FL-Htt-Q145 but not FL-Htt-Q23, compared to controls. These results are consistent with an Hsp70-dependent effect of YM-1 and are in agreement with the selective recognition of mHtt by Hsp70, suggested by the immunoprecipitation analysis, as the effect of YM-1 was genotype-dependent. Interestingly, Hsp90 levels were only altered, being decreased, in cells expressing FL-Htt-Q145 treated with YM-1. An increase in free Hsp90 resulting from the increase in client protein binding to Hsp70 promoted by YM-1, would increase Hsp90 availability and consequently Hsp90 ability to negatively regulate HSF1, inhibiting Hsp90 transcription. Hsp90 is thought to mediate mHtt accumulation through the recruitment of the deubiquitinase Usp19, which deubiquitinates mHtt, decreasing its degradation (He et al., 2016; He et al., 2017). When client proteins can no longer interact with Hsp90, Hsp70 can redirect them for degradation via the UPS (Pratt et al., 2010). This seems to be the case of mHtt, as we only observe a decrease in mHtt levels when we have simultaneously an increase in Hsp70 and its co-chaperone Hsp40, and a decrease in Hsp90 levels. These results are in agreement to those observed in the previous studies where the YM-1 induced decrease on the levels of both the neurodegenerative disorders-associated proteins AR and tau was blocked by proteasomal inhibition (Abisambra et al., 2013; Wang et al., 2013). The UPS is the principal route of protein degradation in mammalian cells (Hipp 2014; Li 2011), thus, alterations of the ubiquitination levels of a subset of misfolded proteins, such as mHtt, may be insufficient to disturb the overall levels of ubiquitinated proteins. Indeed, the levels of ubiquitinated proteins in FL-Htt-Q145 expressing cells were unchanged by the combination of HS and YM-1 treatment compared to solvent conditions.

Treatment with YM-1 increased the levels of mtHsp70 only in FL-Htt-Q145 expressing cells. YM-1 was shown to accumulate in both the cytosolic and mitochondrial fraction of cancer cell models (Koren et al., 2012). mtHsp70 functions in the import and subsequent folding of nucleus-encoded mitochondrial precursor proteins (Chacinska et al., 2009; Schmidt et al., 2010). It is possible that a prolonged interaction of mtHsp70 with its client proteins promoted by YM-1 may alter the dynamic activity of this chaperone, leading to an upregulation of mtHsp70 as perturbations of mitochondrial import can activate the mtUPR (Shpilka and Haynes, 2018). Mitochondrial dysfunction is a key component of HD as mitochondrial bioenergetics, dynamics and quality control are disturbed by the expression of mHtt (Oliveira, 2010b; Guedes-Dias et al., 2016). mHtt was reported to disrupt mitochondrial protein import via direct interaction with TIM23 (Yano et al., 2014). A dysregulation of mitochondrial protein import induced by the expression of mHtt would sensitize these cells to dynamic alterations induced by YM-1, explaining the genotype-dependent effect of YM-1 on mtHsp70 levels. Furthermore, it is also possible that the expression of FL-Htt-Q145 is by itself overwhelming the mitochondrial protein import as

cytosolic aggregation-prone proteins were recently proposed to be imported into mitochondria for degradation by a mechanism termed MAGIC (mitochondria as guardian in cytosol) (Ruan et al., 2017). In this study, the authors showed that aggregation-prone proteins enter the mitochondrial matrix, in yeast, being its degradation delayed by blocking of mitochondrial import (Ruan et al., 2017).

5.3. Molecular chaperone machinery and heat shock response in N-terminal huntingtin expressing cells

Proteolysis of mHtt leads to the generation and accumulation of N-terminal fragments containing the polyQ stretch, which can translocate to the nucleus and are associated with a higher toxicity than full-length Htt (Saudou and Humbert, 2016). To study the proteostasis of N-terminal Htt, we used U2OS cells transfected with EGFP-exon1-Htt-Q23 or EGFP-exon1-HttQ74 construct alone or together with an RFP-β-actin construct, which works as a transfection internal control. Cells expressing NT-Htt-Q23 typically exhibit a diffuse Htt distribution, whereas cells expressing NT-Htt-Q74 exhibit both a diffuse and aggregated Htt distribution. For that reason, we included Hsp110 to the list of Hsp to be evaluated, as Hsp110 plays a role in protein disaggregation. The assessment of Hsp levels by western blot showed that the levels of all the assessed Hsp were unaltered by the expression of NT-Htt-Q74 , compared to NT-Htt-Q23, indicating that the expression of N-terminal mHtt does not activate an HSR or a mtUPR in these cells. As observed in our full-length Htt model, the levels of ubiquitinated proteins in N-terminal mHtt expressing cells were similar to those detected in cells expressing wild-type Htt. Nevertheless, mHtt induced alterations of components of the molecular chaperone machinery may be undetectable due to the low levels of transfected cells versus non-transfected cells.

The evaluation of the Hsp70 distribution by *in situ* immunofluorescence showed that, in cells expressing NT-Htt-Q74, Hsp70 co-localized and accumulated in mHtt aggregates. This could either mean that Hsp70 is being sequestered by mHtt aggregates or that Hsp70 is being recruited to mHtt aggregates to form a chaperone disaggregase complex.

5.4. Effects of Hsp70 modulation in N-terminal huntingtin proteostasis

Treatment with YM-1 alone decreased the levels of soluble NT-Htt-Q74 without altering the levels of soluble NT-Htt-Q23. YM-1 exerted this effect without affecting the levels of the

stress-induced cytosolic Hsp but increasing the levels of the mtHsp70 in both NT-Htt-Q23 and NT-Htt-Q74 expressing cells, reinforcing the hypothesis of an activation of the mtUPR by YM-1 suggested by the previous results of YM-1 effect on mtHsp70 levels in PC12 cells. Regarding aggregated Htt, treatment with YM-1 increased the proportion of cells with mHtt aggregates. Evaluation of the insoluble aggregate load through the quantification of SDS-resistant aggregates showed that, although the mean value of insoluble aggregates is increased in cells treated with YM-1, the levels of these species were not statistically altered by YM-1 treatment. These results on Htt aggregation may seem contradictory, however, not only the imaging and immunoblotting experiments were performed in different conditions (single transfection with EGFP-Htt for imaging versus co-transfection with EGFP-Htt and RFP-β-actin for immunoblotting) but also what is defined as aggregate in each protocol could represent different Htt species. Transfection with more than one plasmid may affect the expression of the exogenous proteins as competition for transcription may occur. In imaging experiments, we simply define as aggregates points of abnormal protein accumulation, independently of their solubility. Thus, these aggregates can include both insoluble species and intermediate soluble species. As the decrease on the soluble levels was not accompanied by an increase on the aggregated levels of mHtt, YM-1 effect is probably associated with an increase in soluble mHtt degradation rather than an alteration of mHtt solubility. Moreover, in this N-terminal Htt model YM-1 effect was not dependent on an increase in both Hsp70 and Hsp40 expression as observed for full-length Htt model, suggesting a differential degradation efficiency of the distinct forms of Htt. Possibly a smaller client protein, such as the truncated form, is more easily targeted for degradation than a bigger client protein, such as the full-length form.

In the cellular model of SBMA, treatment with YM-1 decreased the accumulation of already formed RIPA-insoluble mutant AR aggregates. Again, this effect was attributed to an increase in the protein degradation by the proteasome (Wang et al., 2013). However, while soluble forms of aggregation-prone proteins are primarily targeted to proteasomes for degradation, once they aggregate, they became unable to cross the opening of the proteasome and can only be degraded through autophagy (aggrephagy) (Lamark and Johansen, 2012; Hyttinen et al., 2014). Thus, proteasome-mediated degradation of protein aggregates requires a previous disaggregase activity (Hjerpe et al., 2016). Hsp70 was recently found to be involved in protein disaggregation, acting in cooperation with Hsp40 and the nucleotide exchange factor Hsp110 in the formation of a chaperone complex with disaggregase activity (Nillegoda et al., 2015; Nillegoda et al., 2018). The combined addition of Hsp70, Hsp40 and Hsp110 family members reversed the aggregation of N-terminal mHtt in vitro (Scior et al., 2018). In our N-terminal Htt model, we were unable to consistently

detect Hsp40 and Hsp110 in SDS-resistant aggregates from both solvent and YM-1 treated cells, independently of the levels of mHtt aggregates.

These results suggest that the expression of N-terminal mHtt is not triggering the formation of the disaggregation complex in our model and offer an explanation for why YM-1 is able to decrease soluble mHtt levels without altering the levels of already formed mHtt aggregates. Moreover, treatment with YM-1 did not alter N-terminal mHtt induced cell death, which indicates that the decrease in soluble levels of mHtt was insufficient to ameliorate mHtt toxicity. A longer treatment time with YM-1 may be necessary to induce significant changes in cell death.

5.5. Effects of Hsp70 modulation on mitochondrial function

Results on the levels of mtHsp70 in both cell models suggest that YM-1 treatment can alter mitochondrial function. Taking advantage of the fluorescent properties of YM-1, we assessed its cellular localization by live imaging, confirming its accumulation in mitochondria. MitoTracker Deep Red is a mitochondrial dye that is used as an indicator of mitochondrial membrane potential (Greene et al., 2012; Mot et al., 2016; Xiao et al., 2016). Our preliminary experiments showed a decrease in MitoTracker accumulation in cells expressing NT-Htt-Q74, which was prevented by YM-1. These results suggest that YM-1 can prevent the deficits in mitochondrial membrane potential induced by the expression of mHtt. Indeed, mitochondria isolated from cells of HD patients and brains of HD mice models exhibited deficits in membrane potential (Panov et al., 2002). The decrease in mHtt levels promoted by YM-1 treatment may be enough to reduce the mHtt-induced deficits in mitochondrial membrane potential. Furthermore, an activation of the mtUPR induced by YM-1 treatment could lead to changes of the mitochondrial membrane potential as mtUPR induces the expression of proteins involved in oxidative phosphorylation and ROS production (Shpilka and Haynes, 2018).

6. Conclusion

Molecular chaperones are key elements of the proteostasis network and valuable therapeutic targets in polyglutamine disorders as failure in their activity contributes to the accumulation of misfolded proteins. This study extends the knowledge on the proteostasis of different species of Htt, focusing on how it relates with the chaperone machinery, and evidences the potential of pharmacologically targeting the main effector of this quality control system, Hsp70. The Hsp70 ATPase activity inhibitor YM-1 showed promising results in models of SBMA and tauopathies, however, modulation of specific Hsp may exert different effects depending on the nature of the client protein. The proteostasis of both species is similarly altered by Hsp70 modulation. Treatment with YM-1 increases the degradation of both soluble full-length and N-terminal mHtt, probably via the UPS. Nevertheless, as members of the Hsp70 family can also redirect misfolded proteins for degradation through autophagy-associated pathways, additional studies should be performed in order to clarify the degradation routes involved in YM-1 mediated degradation of mHtt. The absence of improvement in cell survival indicates that the decrease in N-terminal mHtt soluble levels was insufficient to ameliorate mHtt toxicity. Furthermore, preliminary data indicate that YM-1 may affect mitochondrial function, altering mitochondrial membrane potential and increasing the expression of mitochondrial chaperones, possibly through an activation of the mtUPR. Further studies are required to better understand by which mechanisms YM-1 is affecting this organelle that can play an essential role in the activity of the proteostasis network through ATP production. In conclusion, the present study supports pharmacological modulation of Hsp70 as a potential strategy to limit mHtt accumulation in HD and provide new insight on potential off-target effects of YM-1 in specific organelles, such as mitochondria.

CHAPTER VI

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7. References

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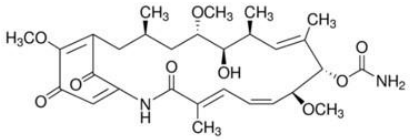
APPENDICES

I. Hsp90 inhibitors



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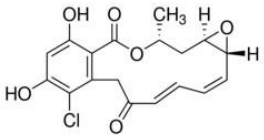

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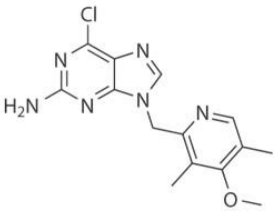

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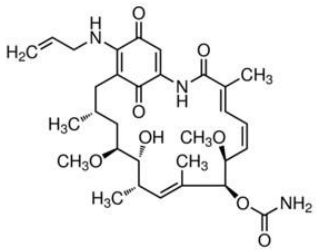

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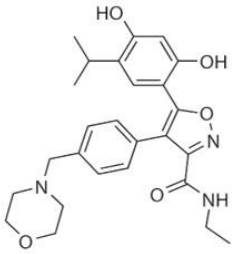

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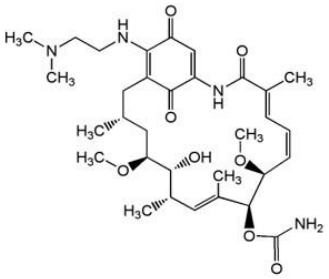

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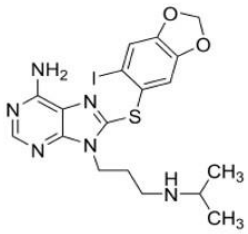

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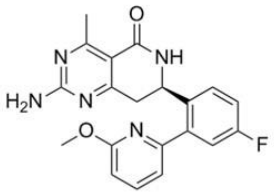

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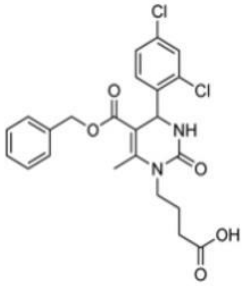
II. Hsp70 modulators

ATPase Activators



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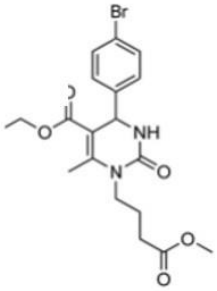

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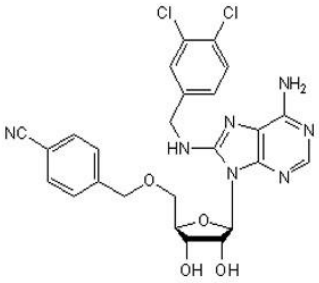
ATPase Inhibitors



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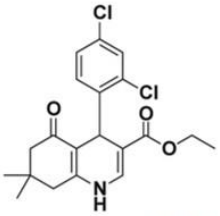

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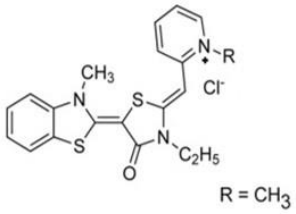

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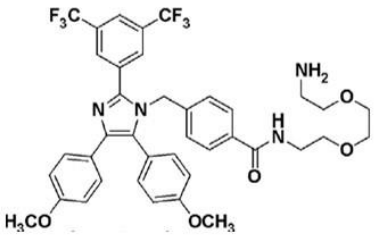

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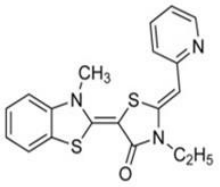

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