LOCALIZATION OF HEAT SHOCK PROTEINS HSPA6 (HSP70B') AND HSPA1A (HSP70-1) IN CULTURED DIFFERENTIATED HUMAN NEURONAL CELLS FOLLOWING THERMAL STRESS

by

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A thesis submitted in conformity with the requirements for the degree of

Doctor of Philosophy

Graduate Department of Cell and Systems Biology, University of Toronto

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ABSTRACT

Localization of Heat Shock Proteins HSPA6 (HSP70B') and HSPA1A (HSP70-1) in Cultured Differentiated Human Neuronal Cells Following Thermal Stress

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Doctor of Philosophy

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2014

Heat shock proteins (Hsps) are a set of highly conserved proteins that are involved in cellular repair and protective mechanisms. In order to identify stress-sensitive sites in differentiated human neuronal cells, cytoplasmic and nuclear localization of two inducible members of the HSPA (HSP70) family was investigated, namely HSPA6 (HSP70B') and HSPA1A (HSP70-1). Stably transfected SH-SY5Y neuronal cell lines were generated that express YFP-HSPA6 and YFP-HSPA1A proteins. Following differentiation, transfected SH-SY5Y cells were exposed to mild heat shock at 43°C for 20 min. Immediately following heat shock, YFP-tagged HSPA6 and HSPA1A proteins localized to centrioles in the cytoplasm and nuclear speckles that are enriched in RNA splicing factors. Subsequently, both stress proteins associated with the granular component of the nucleolus which is the site of ribosomal subunit assembly and ribosomal RNA processing. Later in the recovery period, YFP-HSPA6 protein, but not YFP-HSPA1A, localized to the periphery of nuclear speckles that are sites of RNA transcription and RNA splicing. The present results identify centrioles, nuclear speckles, and the GC component of the nucleolus as potential stress-sensitive sites in differentiated human neuronal cells. The inducible HSPA6 and
HSPA1A proteins exhibit similar localization to these sites. The unique association of the HSPA6 protein with the periphery of nuclear speckles identifies this nuclear site as being stress sensitive, and suggests that the little studied HSPA6 protein may be involved in the recovery of RNA transcription and/or splicing. HSPA6 is a strictly inducible protein, whose gene is present in the human genome but not in rat and mouse. Hence current animal models of neurodegenerative diseases are lacking a potentially protective member of the HSPA family. Sequence and structural analysis revealed that HSPA6 has three additional cysteine amino acids compared to HSPA1A. One of these cysteine amino acids, located in the linker domain, is not found in any other member of the human HSPA family. Identification of stress-sensitive sites in differentiated human neuronal cells could aid the design of therapeutic approaches to protect neurons from the progression of neurodegenerative diseases.
ACKNOWLEDGEMENTS

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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>Degree Celsius</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>APP</td>
<td>Amyloid precursor protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>ATPase</td>
<td>Adenosine triphosphatase</td>
</tr>
<tr>
<td>DB</td>
<td>Digestion buffer</td>
</tr>
<tr>
<td>DFC</td>
<td>Dense fibrillar component</td>
</tr>
<tr>
<td>DNase</td>
<td>Deoxyribonuclease</td>
</tr>
<tr>
<td>EDF</td>
<td>Extended depth of focus</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>Fibrillar centre</td>
</tr>
<tr>
<td>GC</td>
<td>Granular component</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HOP</td>
<td>Hsp70/Hsp90 organizing protein</td>
</tr>
<tr>
<td>Hsp</td>
<td>Heat shock protein</td>
</tr>
<tr>
<td>HSPA</td>
<td>HSP70 family</td>
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<tr>
<td>HSPA1A</td>
<td>HSP70-1 protein</td>
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<tr>
<td>HSPA6</td>
<td>HSP70B' protein</td>
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<tr>
<td>HSR</td>
<td>Heat shock response</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>--------------------------------------------</td>
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<tr>
<td>HSF</td>
<td>Heat shock transcription factor</td>
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<td>HSE</td>
<td>Heat shock element</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LB</td>
<td>Lewy bodies</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>Magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>Minute</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>MPTP</td>
<td>1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium chloride</td>
</tr>
<tr>
<td>NBD</td>
<td>Nucleotide binding domain</td>
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<tr>
<td>NDs</td>
<td>Neurodegenerative diseases</td>
</tr>
<tr>
<td>NEF</td>
<td>Nucleotide exchange factor</td>
</tr>
<tr>
<td>NLS</td>
<td>Nuclear localization signal</td>
</tr>
<tr>
<td>NPC</td>
<td>Nuclear pore complex</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein data bank</td>
</tr>
<tr>
<td>PolyQ</td>
<td>Polyglutamine</td>
</tr>
<tr>
<td>RA</td>
<td>Retinoic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
</tr>
<tr>
<td>RNP</td>
<td>Ribonucleoprotein</td>
</tr>
<tr>
<td>RVC</td>
<td>Ribonucleoside vanadyl complex</td>
</tr>
<tr>
<td>SBD</td>
<td>Substrate binding domain</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the means</td>
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<tr>
<td>SOD-1</td>
<td>Superoxide dismutase-1</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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<tr>
<td>UPS</td>
<td>Ubiquitin-proteasome system</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
</tr>
<tr>
<td>μm</td>
<td>Micrometer</td>
</tr>
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LIST OF PUBLICATIONS

Data presented in this thesis have been published in the following article;


The following manuscript is currently in preparation for publication;

- Localization of HSPA6 (HSP70B’) and HSPA1A (HSP70-1) proteins to nuclear structures following thermal stress in cultured differentiated human neuronal cells.

During my PhD studies, I contributed to other projects that resulted in the following publications from our laboratory;

  (contributed to Figure 4)

  (contributed to Figures 3 and 4)
1 INTRODUCTION

1.1 Heat shock response (HSR)

In response to a range of stressful stimuli, such as temperature elevation, heavy metals, hypoxia and viral infection, cells exhibit a highly conserved heat shock response (HSR) in which general protein synthesis is down-regulated and a set of heat shock proteins (Hsps) is induced (Lindquist and Craig 1988; Pardue et al. 1992; Welch 1992; Feder and Hofmann 1999; Powers and Workman 2007; Richter et al. 2010; Vabulas et al. 2010; Velichko et al. 2013).

In mammalian cells, the induction of Hsps is regulated at the transcription level by heat shock transcription factor-1 (HSF-1) (Wu 1995; Morimoto 1998; Morano and Thiele 1999). Under normal conditions, HSF-1 is present as a monomer. Following stress, HSF-1 is trimerized, phosphorylated, and binds to heat shock elements (HSEs) in the promoter region of stress-inducible heat shock genes, inducing their transcription (Fernandes et al. 1994; Holmberg et al. 2002; Soncin et al. 2003; Guettouche et al. 2005; Kim et al. 2005). Induction of the heat shock response has been reviewed in relation to human aging and neurodegenerative diseases (Anckar and Sistonen 2011; Neef et al. 2011; Heimberger et al. 2013) and upregulation of Hsps suggested as a potential therapeutic approach to counter protein misfolding that underlies neurodegenerative disorders (Selkoe 2004b; Brown 2007b; Haass and Selkoe 2007; Asea and Brown 2008; Brown 2008).
1.2 Heat shock proteins (Hsps)

To minimize cytotoxic effects triggered by the accumulation of misfolded proteins, cells have evolved protein quality control mechanisms that can be categorized as 1) chaperone-mediated folding of nascent and stress-induced misfolded proteins; 2) degradation of misfolded and unfolded proteins by the ubiquitin-proteasome system (UPS); and 3) sequestration and clearance of aggregated proteins through aggresomes when both of the above mentioned strategies fail (Garcia-Mata et al. 2002; Olzmann et al. 2008). Through binding to nascent and misfolded proteins and interaction with co-chaperones, heat shock proteins (Hsps) have been implicated in these protective mechanisms (Zhang and Qian 2011; Dreiseidler et al. 2012; Kim et al. 2013).

The discovery of Hsps occurred with the observation of alteration in chromosome puffing patterns in the chromosomes of Drosophila salivary gland cells following temperature elevation (Ritossa 1962). This was followed by studies on the transcription of heat shock genes and expression of Hsps (Tissieres et al. 1974; Moran et al. 1978). Hsps have been historically referred to as heat shock proteins, however a variety of other stressors induce their expression (Lindquist 1986; Morimoto 1993; Velichko et al. 2013).

Hsps are comprised of both constitutively expressed and stress-inducible members. Constitutive Hsps are involved in cellular housekeeping functions such as protein folding, translocation, and degradation (Lindquist and Craig 1988; Gething and Sambrook 1992; Hartl 1996; Bukau et al. 2006). Small nascent proteins achieve correct folding on their own following translation, however larger polypeptides are dependent on chaperones for proper folding (Kerner et al. 2005). Inducible Hsps are expressed in response to physiological and environmental stimuli to counter stress-induced protein misfolding and aggregation and also to protect against further stresses (Lindquist and Craig 1988; Ellis and van der Vies 1991; Gething and Sambrook 1992;
phenomenon is referred to as ‘thermotolerance’ in which a prior sublethal stress (ie. preconditioning), sufficient to induce the expression of Hsps, protects against a subsequent normally lethal stress (Parsell et al. 1993; Beckham et al. 2008).

Based on their molecular weights, Hsps are classified into Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps (Welch 1993; Saibil 2008; Saibil 2013). Some Hsps interact with members of other families. Hsp40 prevents aggregation of misfolded proteins on its own, but also works as a cochaperone by binding to misfolded proteins and presenting them to Hsp70 for refolding (Greene et al. 1998; Mayer et al. 1999; Fan et al. 2003; Summers et al. 2009). Hsp110 acts as a nucleotide exchange factor for Hsp70 and regulates its function (Dragovic et al. 2006; Shaner and Morano 2007). Hsp70 and Hsp90 bind to each other via the Hsp70/Hsp90 organizing protein (HOP) which coordinates their protein folding activities (Chen and Smith 1998; Wegele et al. 2004).

In a recent high throughput proteomic analysis, 10% of the total protein mass of human tissue culture cells has been found to be composed of chaperone proteins, half of which was Hsp90 and Hsp70 (Finka and Goloubinoff 2013). This highlights the significant contribution of these two classes of Hsps to protein proteostasis in human cells. Given its vital roles in cellular function and protection, HSP70 is the most widely studied HSP multi-gene family (see section 1.4) (Hightower and Li 1994; Mayer and Bukau 2005; Brodsky and Chiosis 2006; Guzhova and Margulis 2006; Daugaard et al. 2007; Goloubinoff and De Los Rios 2007; Morano 2007; Meimaridou et al. 2009).
1.3 Neurodegenerative diseases

Alzheimer’s disease, Parkinson’s disease, Huntington’s disease, and Amyotrophic lateral sclerosis (ALS) are characterized by misfolding and aggregation of proteins and are commonly referred to as “protein misfolding disorders” (Selkoe 2003; Agorogiannis et al. 2004; Ross and Poirier 2004; Muchowski and Wacker 2005; Soto and Estrada 2008).

Extracellular β-amyloid plaques and intracellular neurofibrillary tangles, which are the hallmarks of Alzheimer’s disease, are triggered by the aggregation of cleaved amyloid precursor protein (APP) and hyperphosphorylation of tau proteins, respectively (Selkoe 2004b; Selkoe 2004a; Goedert et al. 2006; Saido 2013). In Parkinson’s disease, formation of α-synuclein-enriched Lewy bodies (LB) in dopaminergic neurons of the substantia nigra is accompanied by the progressive loss of these neurons (Schapira and Olanow 2004; Olanow and Brundin 2013). Abnormal repeats of CAG trinucleotides in the Huntingtonin gene, results in the generation of expanded stretch of glutamine residues, also known as polyQ tracts, which generates a protein that is prone to aggregation. This contributes to the pathogenesis of Huntington’s disease (Gatchel and Zoghbi 2005; Weydt and La Spada 2006; Zheng and Diamond 2012). ALS is characterized by the impairment of motor neurons triggered by the aggregation of ubiquitinated proteins, in particular superoxide dismutase (SOD-1) (Rosen et al. 1993; Banci et al. 2008; Blokhuis et al. 2013).

1.3.1 Protective role of Hsps in neurodegenerative diseases

Due to their demonstrated effects in animal models of human diseases, Hsps have been viewed as both an agent and a target in therapeutic approaches (Sun and MacRae 2005; Kaul and
Neurodegeneration is a devastating age-associated human disease whose frequency is on the rise as average life expectancy has increased. Hsps protect against and delay the progression of neurodegenerative diseases (NDs) by modulating misfolded and aggregation-prone proteins (Barral et al. 2004; Meriin and Sherman 2005; Adachi et al. 2009; Paul and Mahanta 2013). This has led to a quest for agents that can manipulate the heat shock response in order to boost Hsp levels as a therapeutic approach to NDs (Muchowski and Wacker 2005; Brown 2007a; Brown 2007b; Asea and Brown 2008; Brown 2008). The onset of NDs is usually in later stages of life when a diminished induction of Hsps is prevalent (Soti and Csermely 2003; Shamovsky and Gershon 2004; Hung et al. 2010). Our laboratory has previously shown an inverse correlation between the frequency of Alzheimer’s disease, Parkinson’s disease, and ALS, and the abundance of constitutive Hsps in affected neuronal cells (Chen and Brown 2007).

HSP70 family members have been shown to be beneficial in countering protein misfolding and aggregation in ND animal models and tissue culture studies (Patel et al. 2005; Rujano et al. 2007; Turturici et al. 2011). Administration of recombinant Hsp70 increases the life span in the ALS mouse and impedes denervation, which is one of the hallmarks of ALS (Gifondorwa et al. 2007; Gifondorwa et al. 2012). Overexpression of Hsp70 reduces the accumulation of insoluble SOD-1 in an ALS cell culture model (Koyama et al. 2006). In cultured primary motor neurons, Hsp70 overexpression reduces the formation of SOD-containing aggregates (Bruening et al. 1999). Arimoclomol-induced Hsp70 expression decreases protein aggregation and delays disease progression in the ALS mouse model (Kieran et al. 2004; Kalmar et al. 2008; Kalmar et al. 2012).

In transgenic mice, overexpression of Hsp70 suppresses pathological and functional phenotypes of Alzheimer’s disease with lower levels of β-amyloid and decreased neuronal and synaptic loss.
(Hoshino et al. 2011). Upregulation of Hsp70 promotes tau solubility and binding to microtubules in an Alzheimer’s disease model (Dou et al. 2003). Hsps, such as Hsp90, Hsp70, and Hsp32, have been shown to induce the clearance of β-amyloid through phagocytosis in cell culture experiments (Kakimura et al. 2002). Modification of the Hsp70 ATPase activity affects tau stability and has been suggested as a therapeutic approach in Alzheimer’s disease (Jinwal et al. 2010). Using a HIV-derived, cell-penetrating peptide, Tat, delivery of Hsp70 was shown to protect against neuronal cell death in both in vitro and in vivo Parkinson’s disease models (Nagel et al. 2007). Viral delivery of Hsp70 to dopamine neurons of a mouse model of Parkinson’s disease protects them against MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine)-induced neuronal loss (Dong et al. 2005).

1.3.2 SH-SY5Y human neuronal tissue culture cells

Studies on NDs have been conducted in both animal models (Hirsch 2007; Phillips et al. 2009; Avila et al. 2011) and in cell culture models (Figlewicz et al. 2000; Agholme et al. 2010; Byers et al. 2012). SH-SY5Y is an established human neuroblastoma cell line that has been extensively used as a model for NDs including Alzheimer’s disease (Agholme et al. 2010; Zheng et al. 2011; Seidel et al. 2012; Zampagni et al. 2012), Parkinson’s disease (Lopes et al. 2010; Machiya et al. 2010; Xie et al. 2010; Wang et al. 2012), Huntington’s disease (Ding et al. 2002; Banez-Coronel et al. 2012) and ALS (Goos et al. 2007; Pokrishevsky et al. 2012). SH-SY5Y cells were derived from the SK-N-SH cell line which originated from a metastatic neuroblastoma patient (Biedler and Spengler 1976). SH-SY5Y cells can be propagated via mitosis and their differentiation can be induced by treatment with retinoic acid (RA) (Korecka et al. 2013), resulting in a discontinuation of cell division and development of cellular processes. Given their human origin,
SH-SY5Y cells contain the gene coding for Hsp70B′, a novel member of the HSP70 family that is not present in the mouse and rat genomes and hence is absent in current animal models of NDs.

1.4 HSP70 (HSPA) family

HSP70, also known as the HSPA family (section 1.5 on Nomenclature), is a multi-gene family consisting of both constitutively expressed and stress-inducible members that are involved in basic cellular functions namely folding and translocation of cellular proteins (Tavaria et al. 1996; Bukau et al. 2000; Hartl and Hayer-Hartl 2002; Young et al. 2003; De Los Rios et al. 2006; Daugaard et al. 2007; Brocchieri et al. 2008). Expression levels of HSP70 family members have been shown to affect human diseases including cancer (Murphy 2013), autoimmune diseases (Mansilla et al. 2012; Stocki and Dickinson 2012), and neurodegenerative diseases (Brown 2007a; Turturici et al. 2011). Hence members of this family have been viewed as promising agents and targets of therapeutic approaches (Soti et al. 2005; Galluzzi et al. 2009; Jinwal et al. 2010).

HSP70 family members have been highly conserved in evolution and are found in all organisms (Hunt and Morimoto 1985; Lindquist and Craig 1988; Boorstein et al. 1994; Gupta and Singh 1994). HSP70 members can prevent the aggregation of misfolded proteins by binding to them and also facilitate their refolding, which are referred to as ‘holder’ and ‘folder’ activities, respectively (Mayer and Bukau 2005). The latter activity is achieved through ATP-dependent chaperoning cycles, mediated by co-chaperones such as Hsp40 (Fan et al. 2003), in which the HSP70 family member binds to the exposed hydrophobic amino acid stretches of the substrate.
molecules and induces their refolding (Goloubinoff and De Los Rios 2007). Hsp70 is the most abundant stress-inducible HSP found in cells (Welch 1993). In thermotolerant cells, Hsp70 has been shown to be a contributor to cell survival (Johnston and Kucey 1988; Riabowol et al. 1988).

HSP70B′

Hsp70B′ is a strictly stress-inducible member of the HSP70 family that is located on human chromosome 1 (Leung et al. 1990; Parsian et al. 2000). This gene is found in the human genome and is not present in mouse and rat, hence current animal models of neurodegenerative diseases lack a potentially protective member of the HSP70 family (Chow and Brown 2007; Noonan et al. 2007b; Noonan et al. 2008b; Khalouei et al. 2014). Compared to the widely studied Hsp70 (Kiang and Tsokos 1998; Evans et al. 2010; Young 2010; Butler et al. 2013; Jenei et al. 2013; Rauch and Gestwicki 2014), little attention has been given to Hsp70B′. It has been studied in human colon cells (Noonan et al. 2007b; Noonan et al. 2007a; Noonan et al. 2008b; Noonan et al. 2008a), macrophages (Smith et al. 2010), and human SH-SY5Y neuronal tissue culture cells in our laboratory (Chow and Brown 2007; Chow et al. 2010; Khalouei et al. 2014). Hsp70B′ shares 84% protein sequence identity with Hsp70 which will be further reviewed in the Discussion.

1.5 Nomenclature

In the past the nomenclature of HSP70s has been ambiguous with several names referring to a particular member or the same name being used for different members. For example the gene coding for Hsp70 has been referred to as hsp72, hsp70, hsp70-1, hsp70i, and hsx70 by different groups and databases (Tavaria et al. 1996). This ambiguity has been caused by the assignment of
names to HSP70s based on molecular weight, cellular localization, and tissue type expression patterns (Tavaria et al. 1996). The currently accepted HSP70 nomenclature was proposed by Kampinga and colleagues based on gene locus number (Kampinga et al. 2009). In this nomenclature system, the human HSP70 family members have the HSPAX designation (with X corresponding to a particular member) while the corresponding gene names are italicized.

The following annotations are used for heat shock proteins in this thesis:

- The nomenclature proposed by Kampinga et al. 2009, is employed where
  - i) human heat shock proteins are shown in capital letters (e.g. HSPA6) and the corresponding genes are shown in italicized capital letters (e.g. HSPA6)
  - ii) the HSP70 family is designated as HSPA
  - iii) the two human HSPA stress-inducible family members investigated in this thesis, namely HSP70B′ and HSP70-1, are designated as HSPA6 and HSPA1A, respectively
- “Hsp” is used as an abbreviation for heat shock proteins
- In the final reference list, the original nomenclature used in the title of the publication is listed
2 OBJECTIVES

Inducible members of the HSPA (HSP70) family of heat shock proteins are the key players in protecting neurons at times of stress and have been studied in both in vitro and in vivo models (Yenari et al. 1998; Patel et al. 2005; Gifondorwa et al. 2007; Rujano et al. 2007; Turturici et al. 2011). Little attention has been given to HSPA6 compared to other inducible HSPA members, including HSPA1A (Kiang and Tsokos 1998; Evans et al. 2010; Young 2010). The gene coding for the HSPA6 protein is found in the human genome but not in the genomes of rat and mouse, hence current animal models of neurodegenerative diseases are missing this potentially protective heat shock protein.

HSPA members exhibit a high level of sequence similarity and have been shown to exhibit overlapping and distinct features (Daugaard et al. 2007), which could be reflected in their stress-induced intracellular localization. Studies by other groups on the localization of HSPA members have focused on dividing, non-differentiated cells (Liangliang et al. 2010; Kong et al. 2011; Coco et al. 2012; Sabirzhanov et al. 2012; Ogawa et al. 2013). However, neurodegenerative diseases affect the mature human nervous system that is comprised of differentiated neurons.

In this thesis, stress-induced localization of YFP-tagged protein products of HSPA6 and HSPA1A is investigated following thermal stress in differentiated human neuronal SH-SY5Y cell, in order to identify potential stress-sensitive cytoplasmic/nuclear sites and explore similarities and differences in the neuronal localization pattern of HSPA1A and the little studied HSPA6.
The thesis also includes a sequence and structural comparison of HSPA6 and HSPA1A proteins at the level of the nucleotide binding domain, the substrate binding domain, and the linker sequence that connects these two domains.
3 MATERIALS AND METHODS

3.1 Storage and maintenance of SH-SY5Y cells

The human SH-SY5Y neuronal cell line (American Type Culture Collection, Manassas, VA, USA) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37°C in a humidified 5% CO₂ atmosphere.

3.2 Preparation of plasmids

The coding region of human *HSPA1A* was derived from a previously reported *HSPA1A* construct [kind gift from Dr. R. L. Anderson, Peter MacCallum Cancer Centre, Melbourne, Australia; (Chow *et al.* 2009)]. The coding region of *HSPA6* was purchased from RZPD (Berlin, Germany). These coding regions were cloned into a pEYFP-C1 plasmid (Clontech, Palo Alto, CA, USA) fused in-frame with enhanced GFP at the N-terminus by Dr. Ari Chow, Philip Mok, and Erin Chang. The intact pEYFP-C1 vector was used as the control.

3.3 Transformation of bacterial cells and Maxi Prep

JM109 competent cells were transformed with the above mentioned pEYFP-C1 plasmid constructs. Plasmid DNA was extracted using MaxiPrep (QIAGEN). The purity of the extracted DNA was examined by restriction digest and DNA electrophoresis.
3.4 Transfection and selection of SH-SY5Y cells

SH-SY5Y cells constitutively expressing YFP-HSPA6, YFP-HSPA1A or YFP were generated by transfection with the respective YFP fusion construct using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacture’s instruction. The amount of Lipofectamine 2000 and plasmid DNA were optimized. The transfected cells were then selected with 500 µg/ml G418 (Sigma, St Louis, MO, USA) for 6 days. Stable SH-SY5Y cell lines expressing YFP-HSPA6, YFP-HSPA1A, and YFP proteins were then subjected to fluorescence activated cell sorting employing a FACSAria cell sorter (Becton Dickinson, Mississauga, ON, Canada) based on comparable YFP fluorescence levels.

3.5 Differentiation and heat shock of SH-SY5Y cells

Human neuronal SH-SY5Y cells expressing the YFP fusion proteins were plated on coverslips at $3.5 \times 10^4$ cells per cm$^2$ and their differentiation was induced by treatment with 10 µM all-trans-retinoic acid (Korecka et al. 2013) followed by incubation at 37°C for 72 h under serum free conditions. Cells were then heat shocked by immersion in a circulating water bath calibrated at 43°C ± 0.1°C for 20 min and returned to incubation at 37°C. Cells were then harvested at the indicated time points with zero time being the commencement of the heat shock at 43°C.

3.6 Western blotting

Harvested SH-SY5Y cells were solubilized in Laemmli buffer and boiled for 20 min. Following protein quantification by Lowry assay (Lowry et al. 1951), equal loadings of 30 µg of protein per lane were separated by 12% SDS-PAGE and 4% stacking gel, using the Mini-PROTEAN 3
Electrophoresis Module Assembly (Bio-Rad Laboratories, Hercules, CA, USA) and the standard buffer system of Laemmli before transfer to nitrocellulose membranes. Western blotting was performed with antibodies to YFP (clone JL-8, Clontech) for detection of fusion proteins and β-tubulin (MAB3408, Chemicon, Temecula, CA, USA) as loading control. Horseradish peroxidase conjugated secondary antibodies (Sigma) were detected by chemiluminescence assay (Amersham, Piscataway, NJ, USA). Western blots representative of three experimental repeats are shown.

3.7 α-amanitin and RNase/DNase treatment

Human neuronal SH-SY5Y cells expressing the YFP fusion proteins were plated on coverslips and differentiated as outlined in Section 3.5. The transcription inhibitor α-amanitin (ALX-350-270, Enzo Life Sciences) was added to the culture media, 3 h before heat shock at a concentration of 150 μg/ml, and the cells were subsequently fixed at the 3 h time point and coverslips were mounted on slides using Dako fluorescent mounting medium.

RNase and DNase experiments were performed by Dr. Ari Chow and Hashwin Ganesh as follows. Cells grown on coverslips were washed with PBS and lysed on ice for 5 min in CSK buffer (10 mM PIPES (pH 6.8), 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100, 4 mM ribonucleoside vanadyl complex (RVC) (S1402S, New England Biolabs). The coverslips were then rinsed three times in digestion buffer (DB) (10 mM PIPES (pH 6.8), 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5% Triton X-100) and subjected to one of the following treatments at room temperature: 50 μg/ml RNase A (EN0531, Thermo Scientific) in DB (15 min); 50 μg/ml RNase A in DB with RVC (15 min); 150 μg/ml
DNase I (04536282001, Roche Diagnosis) in DB (15 min). The cells were then fixed at the 3 h time point and mounted on slides using Dako fluorescent mounting medium.

3.8 Immunofluorescence

At the indicated time points, cells were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS; pH 7.4) at room temperature for 30 min. Cells were then permeabilized with 0.1% Triton X-100 in PBS containing 100 mM glycine for 30 min, washed, and blocked with 5% FBS in PBS for 2 h. Incubation with primary antibodies was performed in 1% FBS in PBS overnight. Cells were then washed and incubated with fluorescently labeled secondary antibodies, before mounting the coverslip and imaging by fluorescence microscopy using an AxioCam HRm camera with an ApoTome module on an AxioVert 200M microscope (Carl Zeiss, Toronto, ON, Canada), a point-scanning LSM 510 META confocal microscope (Carl Zeiss, Thornwood, NY, USA), or a WaveFX-X1 spinning disc confocal (Quorum Technologies, ON, Canada). The images of RNase/DNase experiments were captured using a spinning disc confocal microscope. For analysis of type III nuclear structures (Figures 7, 8, 9, and 10) extended depth of focus (EDF) of Z-stack planes were generated.

The fluorescence detection of YFP-HSPA6 and YFP-HSPA1A proteins was achieved via the fused YFP tag. For staining of cytoplasmic and nuclear structures, primary antibodies were employed against the centriolar marker proteins γ-tubulin (11-543; Exbio, Prague, Czech Republic) and centrin (04-1624; Millipore, Billerica, MA, USA); the nuclear speckle marker proteins SC35 (ab11826, Abcam, Cambridge, UK) and SON (HPA023535, Sigma-Aldrich, St Louis, MO, USA); the nucleolar marker proteins nucleophosmin (ab10530, Abcam), fibrillarin (ab5821, Abcam), and UBF (sc-13125, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and
RNA Polymerase II (ab5131, Abcam) in combination with donkey anti-mouse Alexa-Fluor 647, donkey anti-rabbit Alexa-Fluor 647, donkey anti-mouse Alexa-Fluor 350, and donkey anti-rabbit Alexa-Fluor 350 secondary antibodies (Invitrogen, Carlsbad, CA, USA). HSPA proteins localized to marker proteins when 80% of the corresponding signal areas overlapped.

3.9 Quantification and statistical analysis

The average counts of three independent experiments (200 cells for each time point) were used for statistical analysis. Data were expressed as the mean ± standard error of the means (SEM). For the quantification of the number of cells that were positive for a centriolar signal (Figure 3A), a two-way analysis of variance (ANOVA) followed by Bonferroni test for pair-wise comparison of means was employed to assess significant differences ($p<0.05$). For the quantification of the number of cells that exhibited type III nuclear structures (Figure 11B), two-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons revealed that the appearance of type III structure in YFP-HSPA6 transfected cells was significantly different from that of YFP-HSPA1A transfected cells ($F = 4248.14$, $DFn = 1$, $DFd = 44$, $p < 0.0001$; * $p < 0.05$ compared to YFP).

3.10 Sequence and structural analysis of HSPA6 and HSPA1A proteins

Protein sequences for human HSPA6 (NP_002146) and HSPA1A (NP_005336) were downloaded from the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein). BioEdit, version 7.2.0. (http://www.mbio.ncsu.edu/bioedit/bioedit.html) (Hall 1999), was used for pairwise alignment using the optimal global alignment based on the Smith and Waterman algorithm (Smith and Waterman 1981). Sequences for human, mouse, and rat HSPA family
members were extracted from the Ensembl database (www.ensembl.org) for the analysis of the conserved linker sequence. PDB files containing the XYZ coordinates of X-ray Crystallography protein structures were downloaded from the Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do). The protein structures files were then opened and color-coded using the PyMol (ver 1.3) molecular visualization program (www.pymol.org).
4 RESULTS

4.1 Stress-induced localization of HSPA6 (HSP70B’) and HSPA1A (HSP70-1) proteins to centrioles in cultured differentiated human neuronal cells

In this thesis, the localization of the YFP-tagged protein products of the \textit{HSPA6} and \textit{HSPA1A} genes is investigated following thermal stress in order to identify potential stress-sensitive sites in the cytoplasm (section 4.1) and nucleus (section 4.2) of differentiated human neuronal cells and determine whether the targeted sites differ for HSPA1A and the little studied HSPA6 proteins.

4.1.1 Characterization of human neuronal cell lines expressing YFP-tagged HSPA6 and YFP-tagged HSPA1A

In order to investigate the localization of HSPA6 (HSP70B’) and HSPA1A (HSP70-1) proteins in human neuronal cells following thermal stress, a strong detectable marker, namely an enhanced yellow fluorescent protein (YFP), was fused to the N-terminus. Transfected cells were selected for transgene expression, subjected to fluorescence activated cell sorting and stable cell lines generated (Figure 1A). Western blot analysis demonstrated that cell lines expressing YFP-tagged HSPA6, YFP-tagged HSPA1A and YFP alone were obtained (Figure 1B).
Figure 1. Characterization of human neuronal cell lines stably expressing YFP-tagged HSPA6 (HSP70B′) and YFP-tagged HSPA1A (HSP70-1) proteins

A) Stable SH-SY5Y cell lines expressing YFP-HSPA6, YFP-HSPA1A and YFP proteins were enriched by fluorescence activated cell sorting employing a FACSARia cell sorter (Becton Dickinson, Mississauga, ON, Canada) based on comparable YFP fluorescence levels. B) Western blot of neuronal cell lines expressing YFP-HSPA6, YFP-HSPA1A and YFP proteins. NT: non-transfected cells. Western blotting was performed with antibodies to YFP (clone JL-8, Clontech) for detection of fusion proteins and β-tubulin (MAB3408, Chemicon, Temecula, CA, USA) as a loading control. A Western blot representative of three experimental repeats is shown.
4.1.2 YFP-HSPA6 and YFP-HSPA1A at centrioles in differentiated human neuronal cells following thermal stress

Immediately following thermal stress at 43°C for 20 min, YFP-tagged HSPA6 and HSPA1A were detected in prominent structures resembling centrioles in the cytoplasm of post-mitotic, differentiated human neurons (Figure 2A). In controls, the YFP-tagged proteins were diffuse throughout the cytoplasm. After thermal stress, YFP-HSPA1A was present at putative centrioles at the 1 h time point. In contrast, YFP-HSPA6 persisted at putative centrioles for longer time periods and was still apparent at 3 h. As shown in Figure 2B and C, the cytoplasmic structures that were positive for YFP-tagged HSPA6 and HSPA1A, localized with a centriole marker protein. The appearance of HSPA6 and HSPA1A at centrioles was rapid but transient after thermal stress, as centriole-associated signal was not present at 4 h (Figure 2A, B and C). Centrioles were present at the 4 h as evidenced by the centriole marker (Figure 2B and C). The YFP control cell line did not exhibit a centriole-associated signal despite the presence of centrioles at both 1 h and 4 h as identified by the centriole marker (Figure 2D).

4.1.3 Prolonged association of YFP-HSPA6 with the proximal end of centrioles

The observation of a prolonged YFP-HSPA6 signal at centrioles was confirmed by quantification of the number of cells that were positive for centriole signal as shown in Figure 3A. At 2 h, 87% of the neuronal cells in the YFP-HSPA6 line were positive for a signal in centrioles, whereas only 31% were positive in the YFP-HSPA1A cell line. At 3 h, the percentage of cells positive for a centriole signal was 34% and 2%, respectively. Analysis of neuronal proteins isolated at time
Figure 2. YFP-HSPA6 and YFP-HSPA1A proteins localize to centrioles in differentiated human neuronal cells following thermal stress

A) Time course of YFP-HSP70 proteins at putative centrioles after heat shock at 43°C for 20 min and recovery at 37°C. White arrows show YFP-HSPA6 at putative centrioles for longer time periods compared to YFP-HSPA1A. B) YFP-HSPA6 positive cytoplasmic structures localized with the centriole marker, γ-tubulin. In control and 4 h time points, a YFP-HSPA6 positive cytoplasmic signal was not observed despite the presence of the centriole marker signal. C) YFP-HSPA1A positive cytoplasmic structures also localized with the centriole marker at the 1 h time point. D) The YFP control cell line did not exhibit a YFP signal associated with centrioles. Scale bars represent 5 μm.
Figure 3. Prolonged association of YFP-HSPA6 protein with the proximal end of centrioles

A) Quantification of the number of cells that were positive for a centriolar signal in YFP-HSPA6, YFP-HSPA1A and YFP transfected cell lines. Both YFP-HSPA proteins appear rapidly at centrioles, however, YFP-HSPA6 remained at centrioles for up to 3 h, whereas YFP-HSPA1A did not. B) Western blot analysis of YFP-HSPA6 and YFP-HSPA1A levels at time points following thermal stress. Prolonged association of YFP-HSPA6 with centrioles compared to YFP-HSPA1A was not due to degradation of YFP-HSPA1A. NT: non-transfected. A Western blot representative of three experimental repeats is shown. C) Localization of YFP-HSPA6 to the proximal end of centrioles. YFP-HSPA6 localized with γ-tubulin, a marker of the proximal end of centrioles and not with centrin, a marker of the distal end. D) Localization of YFP-HSPA1A to the proximal end of centrioles. Similar to YFP-HSPA6 protein, YFP-HSPA1A localized with γ-tubulin and not with centrin. Scale bars represent 5 µm.
points after thermal stress indicated that levels of YFP-HSPA6 and YFP-HSPA1A did not change, suggesting that the prolonged YFP-HSPA6 signal at centrioles compared to that of YFP-HSPA1A, was not due to degradation of YFP-HSPA1A (Figure 3B).

Each component of the doublet centriole has a distal and a proximal end (Azimzadeh and Marshall 2010; Bornens 2012). The localization of YFP-tagged proteins was investigated at higher magnification using marker proteins of either the distal (centrin) or the proximal end (γ-tubulin) of the centriole (Bornens 2002; Brito et al. 2012). YFP-HSPA6 and YFP-HSPA1A localized with γ-tubulin rather than centrin (Figure 3C and D), suggesting that the two proteins associate with the proximal rather than the distal end of the centriole.

4.2 Localization of HSPA6 and HSPA1A proteins to nuclear structures following thermal stress in cultured differentiated human neuronal cells

Heat shock proteins (Hsps) are a group of highly conserved proteins that protect cells against physiological and environmental stresses (Lindquist and Craig 1988; Ellis and van der Vies 1991; Gething and Sambrook 1992; Georgopoulos and Welch 1993; Morimoto et al. 1994). Analysis of the subcellular localization of Hsps following thermal stress was employed to identify stress-sensitive sites in differentiated human neuronal cells. In this study the nuclear localization of HSPA6 and HSPA1A, two stress-inducible members of the HSPA family is investigated to explore whether they exhibit similar or different sites of nuclear localization following stress. Stable cell lines expressing YFP-tagged HSPA6 and HSPA1A proteins were generated and their nuclear localization at specific time points following mild heat stress (43°C
for 20 min) was examined using fluorescence microscopy. A rapid and transient localization of both stress-inducible heat shock proteins to nuclear speckles was observed, followed by localization to the granular component of the nucleolus. Later in the recovery period, at the 3, 5, and 7 h time points, YFP-HSPA6 protein exhibited a distinct localization at the periphery of nuclear speckles which was not observed in YFP-HSPA1A transfected cells.

4.2.1 YFP-HSPA6 and YFP-HSPA1A proteins associate with nuclear components of differentiated human neuronal cells after heat shock

Following heat shock at 43°C for 20 min and during the subsequent recovery period at 37°C, a localization of YFP-HSPA6 to nuclear components was observed (Figure 4). Type I structures were observed immediately following heat shock. At the 2 h time point, donut-shaped type II structures appeared. Later in the recovery period at the 3, 5, and 7 h time points, but not at 24 h, bright type III structures were observed.

Following thermal stress, YFP-HSPA1A protein showed a similar pattern of localization to nuclear type I and type II structures, however, type III structures were not apparent (Figure 4). Type I, II, and III structures were not observed in the YFP control cell line (Figure 4). In order to characterize these nuclear structures, a series of immunofluorescence experiments were conducted as outlined in the following sections.
Following heat shock at 43°C for 20 min, YFP-HSPA6 localized to type I nuclear structures. At the 2 h time point during the recovery incubation at 37°C, YFP-HSPA6 localized to donut-shaped type II nuclear structures. Later in the recovery period at the 3, 5, and 7 h time points, but not at 24 h, YFP-HSPA6 localized to bright type III structures. Type I and II, but not type III nuclear structures, were observed in YFP-HSPA1A transfected cells. The YFP control cell line did not exhibit type I, II, and III structures following heat shock or during the recovery period. The white arrows in the YFP-HSPA6 and YFP-HSPA1A panels indicate representative nuclear structures. Scale bar represents 5 μm.
4.2.2 Transient localization of YFP-HSPA6 and YFP-HSPA1A proteins with nuclear speckles (type I structures)

Following the 20 min heat shock period, YFP-HSPA6 protein was observed to be associated with type I structures that localized with the nuclear speckle marker proteins SC35 and SON (Figure 5A and B, respectively). Nuclear speckles are sites enriched in RNA splicing factors and active transcription takes place at their periphery (Lamond and Spector 2003; Brown et al. 2008; Spector and Lamond 2011). SC35 and SON have been previously used as nuclear speckle markers (Salsman et al. 2008; Sharma et al. 2010; Sytnikova et al. 2011). As shown in Figure 5C, SC35 and SON localized with each other and with the type I YFP-HSPA6 signal. Localization of type I structures with the nuclear speckle marker SC35 was also observed in YFP-HSPA1A transfected cells immediately after heat shock (Figure 5D). The YFP protein in control cells did not exhibit type I structures (Figure 5E).

4.2.3 YFP-tagged HSPA6 and HSPA1A proteins associate with the granular component (GC) of nucleolus (type II structures)

At the 2 h time point following thermal stress, YFP-tagged HSPA6 protein localized to donut-shaped type II nuclear structures (white arrow in Figures 4 and 6A) that were distinct from type I structures identified by the nuclear speckle marker SC35. These type II structures appeared to be localized at the nucleolus, a prominent nuclear structure that has been studied extensively (Lam et al. 2005; Thiry and Lafontaine 2005; Raska et al. 2006; Sirri et al. 2008; Hernandez-Verdun et al. 2010). The nucleolus is composed of three main components (Figure 6B). The innermost fibrillar centre (FC) is the site of transcriptionally inactive ribosomal RNA (rRNA) genes; the
Figure 5. Transient localization of YFP-HSPA6 and YFP-HSPA1A proteins to nuclear speckles (type I structures)

A and B) Following heat shock (43°C for 20 min), the YFP-HSPA6 protein associated with type I structures that localized to the nuclear speckle markers SC35 and SON, respectively. C) Nuclear speckles were identified by SC35 and SON, which localized with each other and with the YFP-HSPA6 positive nuclear type I structures. D) Type I structures in YFP-HSPA1A transfected cells exhibited a similar localization with the nuclear speckle marker SC35 immediately following the 20 min heat shock period. E) Type I structures were not observed in YFP control cells following heat shock. Scale bar represents 5 μm.
Figure 6. YFP-tagged HSPA6 and HSPA1A proteins associate with the granular component (GC) of the nucleolus (type II structures)

A) At the 2 h time point following thermal stress, YFP-HSPA6 protein localized to donut-shaped type II nuclear structures (white arrow) that were distinct from type I nuclear structures identified by the nuclear speckle marker protein SC35. B) The three components of the nucleolus, namely the fibrillar centre (FC; innermost), dense fibrillar component (DFC), and granular component (GC; surrounding the other two components), can be identified by the marker proteins nucleophosmin, fibrillarin, and UBF, respectively. C) YFP-HSPA6 protein associated with type II structures that localize to the GC component of nucleolus, identified by nucleophosmin. D and E) Type II nuclear structures did not localize to the DFC and FC components of the nucleolus, identified by fibrillarin and UBF, respectively. F to H) Analysis of the heat shocked YFP-HSPA1A transfected cells showed that, similar to the YFP-HSPA6 transfected cells, YFP-HSPA1A protein associated with type II structures that localized to the GC component of nucleolus, identified by nucleophosmin (F) and not to the DFC or FC components (identified by fibrillarin and UBF, respectively) (G and H). Type II structures were not observed in YFP control cells following heat shock (I). Scale bars represent 5 μm.
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dense fibrillar component (DFC), surrounding the FC component, is a zone of active rRNA transcription; and the outermost granular component (GC) is involved in rRNA processing and ribosomal subunit assembly (Lam et al. 2005; Thiry and Lafontaine 2005; Raska et al. 2006; Sirri et al. 2008; Hernandez-Verdun et al. 2010). The FC, DFC, and GC components of the nucleolus can be identified by the marker proteins, UBF, fibrillarin, and nucleophosmin, respectively (Figure 6B). As shown in Figure 6C, the YFP-HSPA6 positive type II structures localized to the GC component of the nucleolus as identified by the nucleophosmin marker protein. Type II nuclear structures did not localize to the DFC or FC components of the nucleolus, identified by fibrillarin and UBF marker proteins (Figure 6D and E).

YFP-HSPA1A protein also associated with type II nuclear structures at the 2 h time point that localized to the GC component of the nucleolus, identified by the nucleophosmin marker protein (Figure 6F). Furthermore, type II structures in YFP-HSPA1A transfected cells did not localize to the DFC or FC components of the nucleolus as identified by fibrillarin and UBF, respectively (Figure 6G and H). Type II nuclear structures were not observed in the YFP control cell line following heat shock (Figure 6I).

4.2.4 Localization of YFP-HSPA6 protein to the periphery of nuclear speckles (type III structures)

Later in the recovery period at the 3, 5, and 7 h time points after thermal stress, YFP-HSPA6 protein localized to type III structures (Figure 4) that were clustered at the periphery of nuclear speckles that were identified by SC35 and SON (Figure 7A and B, respectively). Type III nuclear structures were not observed in YFP-HSPA1A transfected cells at any of the time points.
Figure 7. Localization of YFP-HSPA6 proteins to the periphery of nuclear speckles (type III structures)

A and B) Following heat shock (43°C for 20 min) at the 3 h recovery time point, YFP-HSPA6 protein localized to type III structures at the periphery of nuclear speckles (identified by SC35 and SON). The white boxes correspond to the magnified areas shown to the right. C) Type III structures were not observed in the YFP-HSPA1A transfected cells following heat shock at the 3 h recovery time point. D) Type III nuclear structures were not observed in the YFP control cell line. Scale bars represent 5 μm.
following heat shock (Figure 4 and Figure 7C). These type III structures were not observed in the YFP control cell line (Figure 4 and Figure 7D).

4.2.5 Type III structures at the periphery of nuclear speckles that is enriched in RNA polymerase II

The periphery of nuclear speckles has been reported to be a zone of active gene transcription that is enriched in poly(A)-containing RNA (Hall et al. 2006) and RNA polymerase II (Bregman et al. 1995; Mortillaro et al. 1996). Figure 8 indicates that at the 3 h time point following heat shock, YFP-HSPA6 protein localized to type III structures at the periphery of SC35-positive nuclear speckles that is enriched in RNA polymerase II.

4.2.6 Effect of α-amanitin on the localization of YFP-HSPA6 protein to type III nuclear structures

α-Amanitin, a potent transcription inhibitor extracted from the mushroom Amanita phalloides, blocks the activity of RNA polymerase II (Lindell et al. 1970; Nguyen et al. 1996). It has been used to inhibit transcription in culture cell studies including SH-SY5Y cells (Tokunaga et al. 2006; Meguro-Horike et al. 2011; Vilotti et al. 2012; Mita et al. 2013). Treatment of cells with α-amanitin results in the enlargement and rounding of nuclear speckles (Kumaran et al. 2002; Tabellini et al. 2003; Xie et al. 2006).

As shown in Figure 9, α-amanitin treatment resulted in the enlargement of SC35/SON-positive nuclear speckles and YFP-HSPA6 type III structures remain clustered at the periphery of the
Figure 8. Type III structures at the periphery of nuclear speckles that is enriched in RNA polymerase II

Following heat shock at the 3 h recovery time point, YFP-HSPA6 protein associated with type III structures at the periphery of SC35-positive nuclear speckles that is enriched in RNA polymerase II. Scale bar represents 5 μm.
Figure 9. Effect of α-amanitin on YFP-HSPA6 positive type III structures at the periphery of nuclear speckles

The addition of α-amanitin resulted in the enlargement and rounding of nuclear speckles (identified by SC35 and SON). YFP-HSPA6 positive type III structures remained clustered at the periphery of the enlarged nuclear speckles suggesting they were tethered at the periphery of nuclear speckles. Scale bar represents 5 μm.
enlarged nuclear speckles. These results suggest that the HSPA6-positive type III structures are tethered at the periphery of nuclear speckles.

4.2.7 Type III nuclear structures are RNase-sensitive

The periphery of nuclear speckles is enriched in both poly(A)-containing RNA (Hall et al. 2006) and RNA polymerase II (Bregman et al. 1995; Mortillaro et al. 1996) and it is a platform for active RNA transcription and splicing (Wei et al. 1999; Spector and Lamond 2011). The localization of HSPA6 positive type III structures around the periphery of nuclear speckles suggests that type III structures may be related to synthesis and/or processing of RNA molecules. In order to investigate this, YFP-HSPA6 cells were treated with RNase and DNase. The type III nuclear structures that were present in YFP-HSPA6 cells at the 3 h time point after heat shock, were sensitive to RNase but not DNase digestion (Figures 10A and B, respectively). These results suggest that the type III nuclear structures may be involved in the synthesis and/or processing of RNA molecules.

4.2.8 Comparison of type III nuclear structures in YFP-HSPA6 and YFP-HSPA1A transfected cells

As shown in Figure 11A, the type I and type II nuclear structures were observed in both YFP-HSPA6 and YFP-HSPA1A transfected cell lines. However, the type III nuclear structures were observed in YFP-HSPA6 transfected cells but not in YFP-HSPA1A transfected cells. A quantification of the number of cells positive for type III structures indicated that 73% of the YFP-HSPA6 transfected cells exhibited type III nuclear structures, but less than 1% of the YFP-
Figure 10. Type III nuclear structures are RNase-sensitive

HSPA6 positive type III nuclear structures that were present in heat shocked cells, at the 3 h recovery time point, were sensitive to digestion with RNase (A) but not DNase (B). Nuclear speckles are identified by the SC35 and SON marker proteins. Scale bars represent 5 µm.
Figure 11. Comparison of type III nuclear structures in YFP-HSPA6 and YFP-HSPA1A transfected cells

A) Type I and II nuclear structures are observed in YFP-HSPA6 and YFP-HSPA1A transfected cell lines but type III nuclear structures are observed only in YFP-HSPA6 transfected cells. B) Quantification of the number of cells that exhibit type III structures, showed that these nuclear structures are observed in 73% of the YFP-HSPA6 transfected cells but in less than 1% of YFP-HSPA1A transfected cells (averaged over the 3 h to 7 h time points). Three independent experiments were conducted and in each experiment, two sets of 200 cells were counted. Data were expressed as the mean ± standard error of the means (SEM). Type III nuclear structures were not observed in YFP control cells. Two-way analysis of variance (ANOVA) followed by Bonferroni’s test for multiple comparisons were performed as described in Materials and Methods. C) Western blot analysis of protein levels, using an anti-YFP antibody showed that levels of YFP-HSPA6 and YFP-HSPA1A proteins did not change at time points following heat shock. β-tubulin was used as loading control. A Western blot representative of three experimental repeats is shown. Scale bar represents 5 μm.
C

Anti-YFP

Anti-β-tubulin

Anti-YFP

Anti-β-tubulin
HSPA1A transfected cells (Figure 11B). Western blot analysis of protein levels at time points following thermal stress showed that the levels of YFP-HSPA6 and YFP-HSPA1A proteins did not change, indicating that the absence of type III nuclear structures in YFP-HSPA1A transfected cells is not due to the degradation of YFP-HSPA1A protein following heat shock (Figure 11C).

Overall, YFP-HSPA6 and YFP-HSPA1A proteins exhibited similarities and differences in their stress-induced localization in the cytoplasm and nucleus of differentiated human SH-SY5Y neuronal cells. As previously mentioned, HSPA6 is found in the human genome and not in mouse and rat. A longer association of YFP-HSPA6 was observed at the proximal end of centrioles in the cytoplasm following thermal stress, compared to YFP-HSPA1A.

Within the nucleus, both proteins localized to the nuclear speckles (type I structure) and the GC component of nucleolus (type II structure) following thermal stress, however later in the recovery period at the 3 h time point, YFP-HSPA6 protein localized to the periphery of nuclear speckles (type III structure) which was not observed in YFP-HSPA1A cells. The following sections include an analysis of sequence differences between HSPA6 and HSPA1A proteins and the position of the different amino acids in the protein structure.
4.3 **Sequence and structural analysis of HSPA6 and HSPA1A proteins**

4.3.1 **Sequence analysis of HSPA6 and HSPA1A proteins**

The protein domains in HSPA members will first be described and how they are organized into protein tertiary structures. This will be followed by a protein sequence comparison of HSPA6 and HSPA1A. HSPA members are composed of two main domains; an N-terminal nucleotide binding domain (NBD), also known as the ATPase domain, and a C-terminal substrate binding domain (SBD). A short conserved linker region connects these two domains. The NBD and SBD domains are shown as sequence blocks in Figure 12A and with the same color coding as the protein structure of the bacterial Hsp70 homologue, DnaK, shown in Figure 12B. Only the NBD domain has been crystalized for HSPA6 and HSPA1A proteins (Sriram et al. 1997; Shomura et al. 2005; Wisniewska et al. 2010; Arakawa et al. 2011). The full protein structure of DnaK is available (Bertelsen et al. 2009) and has been used in the literature for structural and functional analysis of HSPA members (Kampinga and Craig 2010; da Silva and Borges 2011; Chiappori et al. 2012; Golas et al. 2012; Zhuravleva et al. 2012; Clare and Saibil 2013; Mayer 2013) and will be used as a reference structure in this thesis.

The NBD domain is composed of two lobes; designated as I and II. Each lobe, in turn is divided into two subdomains; A and B (Zhuravleva and Gierasch 2011) (Figures 12A and B). The IA, IB, IIA, and IIB domains are highlighted in dark green, orange, purple, and blue, respectively. These lobes create a deep nucleotide binding cleft (Figure 12B). A terminal α-helix at the C-terminus of the NBD domain (shown in red) is followed by the conserved linker sequence (shown in cyan) which connects the NBD to the SBD domain. The SBD is divided into a β-sheet segment (β-SBD) (shown in burgundy) that forms a substrate binding cleft and an α-helix.
Figure 12. HSPA (HSP70) functional domains

A) The protein structure of HSPA (HSP70) members are divided into two main domains; the nucleotide binding domain (NBD), where the ATP nucleotide binds and the substrate binding domain (SBD) which is subdivided into β-SBD containing beta sheets that provide a binding site for the substrate and a α-SBD alpha-helical lid that closes on the substrate upon the hydrolysis of the ATP to ADP. A short conserved linker binds the two domains. The C-terminus ends in a highly conserved EEVD motif. B) The protein structure of the bacterial Hsp70 homologue, DnaK, is one of the few Hsp70 protein structures that is crystalized as a whole (Bertelsen et al. 2009) (PDB id: 2KHO) which has been used in this thesis as a reference structure. The nucleotide binding domain (NBD) and substrate binding domain (SBD) are connected by a conserved linker domain (cyan). Lobes I and II are shown on the NBD domain along with their subdomains A and B. In the ADP-bound state, the α-helical lid of the SBD domain closes on the substrate binding cleft as shown in the figure.
domain (α-SBD) (shown in green) which closes as a “lid” on the substrate upon the hydrolysis of ATP in the NBD domain (Zhu et al. 1996; Bertelsen et al. 2009; Mayer 2010; Young 2010; Chiappori et al. 2012) (Figures 12A and B).

HSPA6 and HSPA1A protein sequence alignment

Figure 13 shows a pair-wise alignment of HSPA6 and HSPA1A sequences using the same color coding of different domains that was employed in Figures 12A and B. The HSPA6 sequence is composed of 643 amino acids. The second and third amino acids at the N-terminus (Glu2 & Ala3) of the HSPA6 sequence are not present in HSPA1A, resulting in a 641 amino acid sequence. There is 84% identity between the two sequences, with the majority of differences occurring in the α-helical region of the substrate binding domain (α-SBD; green). There are 49 amino acid differences in the NBD domain, 2 in the linker sequence and 64 in the SBD domain (ie. 7 in the β-SBD and 57 in the α-SBD domain). The conserved substitutions are indicated by a plus sign as determined by the NCBI Blast server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Other annotations (e.g. purple circles and yellow triangles) correspond to features discussed in the upcoming sections.

4.3.2 Mechanism of action of HSPA (HSP70) proteins

HSPA (HSP70) proteins are involved in the ATP-dependent folding of misfolded proteins (Mayer and Bukau 2005; Saibil 2008; Summers et al. 2009; Young 2010; Saibil 2013). Hsp70 function is regulated by its co-chaperone Hsp40 and nucleotide exchange factors (NEF’s) (Hartl and Hayer-Hartl 2009; Mayer 2010). This process begins by binding of the misfolded protein to
The numbering is based on the HSPA6 sequence and will be used when referring to the amino acid positions on the protein sequence or structures throughout this thesis. Only the amino acid differences are shown in the HSPA1A sequence, with the conserved substitutions (e.g. R27K) designated by a ‘+’ sign beneath as identified by the NCBI BLAST server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The alignment and graphical output was obtained using BioEdit 7.2.0. as described in Materials and Methods. The following annotations were used which will be discussed in upcoming sections: purple box: conserved EEVD motif; blue lines on top of amino acid in β-SBD domain: amino acids in the proximity of bound substrate; purple circles in α-SBD domain: amino acids on the α-helical domain facing the bound substrate; yellow triangles: the eight cysteine residues in HSPA6 and HSPA1A sequences (the three triangles having a star inside corresponds to those absent in HSPA1A).
the Hsp70 co-chaperone, Hsp40, which delivers this substrate to the Hsp70 SBD domain (Cyr et al. 1994; Summers et al. 2009) (Figure 14; Stage 1). In addition to the substrate delivery, Hsp40 also facilitates the hydrolysis of ATP to ADP by binding to the NBD domain of Hsp70 via its J-domain (Cyr et al. 1994; Walsh et al. 2004; Qiu et al. 2006; Jiang et al. 2007; Hartl and Hayer-Hartl 2009; Mayer 2010).

Conversion of ATP to ADP results in conformational changes in the NBD domain which is allosterically communicated to the SBD domain via the linker sequence (da Silva and Borges 2011; Zhuravleva et al. 2012). This in turn results in the locking of the α-helical lid domain of the SBD and tight binding of the substrate (Chang et al. 2008; Bertelsen et al. 2009; Mapa et al. 2010) and also ‘undocking’ of the two domains (Swain et al. 2007) (Figure 14; Stage 2). The exposed hydrophobic amino acids of the non-native protein, which are normally buried within the native protein structure, bind to the SBD cleft in an extended conformation (Bukau and Horwich 1998; Daugaard et al. 2007; Patury et al. 2009; Mayer 2010). In this ADP-bound state, there is a high substrate affinity and a low exchange rate for the substrates (Mayer 2010).

The variable C-terminus domain has also been suggested to confer specificity to different HSPA members (Daugaard et al. 2007). The EEVD motif at the end of the α-helical domain is implicated in both ATPase activity and interaction with substrate and Hsp40 (Freeman et al. 1995) (Figure 13). The binding of NEFs, such as BAG and Hsp110, facilitate the ADP release by inducing conformational changes in the NBD domain (Sondermann et al. 2001; Polier et al. 2008; Schuermann et al. 2008; Xu et al. 2008; Arakawa et al. 2010) (Figure 14; Stage 3). Subsequently, the release of the NEF and binding of a new ATP molecule brings about destabilization of regions around the substrate binding site causing a weaker affinity and higher association and dissociation rates for the substrate (Swain et al. 2007; Mayer 2010), with a
Figure 14. Mechanism of action of HSPA (HSP70) proteins

In the ATP-bound state, the lid domain of SBD domain adopts an open conformation, exposing the substrate binding site within the cleft. Hsp40, a cochaperone of Hsp70, delivers the non-native protein to the SBD domain (stage 1) and also induces the hydrolysis of ATP to ADP, which results in the locking of the lid domain on the substrate (stage 2). Binding of a nucleotide exchange factor (NEF) to the NBD domain releases the ADP (stage 3). Finally, NEF dissociates from the NBD domain and binding of an ATP molecule induces conformational changes in the NBD domain which through allosteric communications causes the opening of the lid and the release of the refolded substrate (stage 4) (Mayer and Bukau 2005; Summers et al. 2009).
concomitant opening of the α-helical lid domain (Liu and Hendrickson 2007; Kityk et al. 2012; Zhuravleva et al. 2012). These changes results in the release of the substrate from the SBD domain (Figure 14; Stage 4). Binding of the ATP stabilizes the NBD domain and destabilizes the SBD domain (Buchberger et al. 1995; Rist et al. 2006) and based on the observations from available protein structures has been proposed to induce the formation of a tighter complex and ‘docking’ of the NBD and SBD domains (Swain et al. 2007).

In the next section, the observed amino acid differences between HSPA6 and HSPA1A sequences are analyzed in the three domains of the protein structures, namely NBD domain, SBD domain, and the linker sequence connecting the two domains.

4.3.3 Structural analysis of the HSPA6 and HSPA1A proteins

The RCSB Protein Data Bank (PDB) (http://www.rcsb.org/pdb/home/home.do), is the main repository site for X-ray crystallography, NMR and electron microscopy protein structures, with more than 84000, 10000, and 650 structures in each category, respectively. There has been a significant increase in the determination of protein structures of HSPA family members in the past two decades which has increased our understanding of their mechanism of action. For human HSPA1A protein, there are multiple structures of the NBD domain for ADP, ATP, and NEF bound states (Sriram et al. 1997; Osipiuk et al. 1999; Shomura et al. 2005; Arakawa et al. 2011). The only structure available for the HSPA6 protein corresponds to the NBD domain (Wisniewska et al. 2010). However, as previously mentioned, there are full structures available for the bacterial Hsp70 homologue, DnaK, in both closed (ADP-bound with substrate) (Bertelsen et al. 2009) and open (ATP-bound) conformations (Kityk et al. 2012). Therefore the DnaK structure was used for reference in this thesis. Table 1 shows a representative list of these
Table 1. Protein data bank identifier codes for HSPA6, HSPA1A, and DnaK protein structures

This list contains the only available HSPA6 structure and representative structures of HSPA1A and DnaK, along with the corresponding domain and reference.
<table>
<thead>
<tr>
<th>Protein</th>
<th>PDB</th>
<th>Molecule</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
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<td>3FE1</td>
<td>NBD</td>
<td>(Wisniewska et al. 2010)</td>
</tr>
<tr>
<td>HSPA1A</td>
<td>1S3X</td>
<td>NBD</td>
<td>(Sriram et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>1HJO</td>
<td>NBD</td>
<td>(Osipiuk et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>1XQS</td>
<td>NBD (partial)</td>
<td>(Shomura et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>3LOF</td>
<td>SBD C-terminal domain</td>
<td>NA</td>
</tr>
<tr>
<td></td>
<td>3ATU</td>
<td>NBD</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3ATV</td>
<td>NBD</td>
<td>(Arakawa et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>3AY9</td>
<td>NBD</td>
<td></td>
</tr>
<tr>
<td>DnaK</td>
<td>1DKZ</td>
<td>SBD</td>
<td>(Zhu et al. 1996)</td>
</tr>
<tr>
<td></td>
<td>1DKG</td>
<td>NBD</td>
<td>(Harrison et al. 1997)</td>
</tr>
<tr>
<td></td>
<td>2KHO</td>
<td>Full structure (closed)</td>
<td>(Bertelsen et al. 2009)</td>
</tr>
<tr>
<td></td>
<td>4B9Q</td>
<td>Full structure (open)</td>
<td>(Kityk et al. 2012)</td>
</tr>
</tbody>
</table>
structures along with their PDB access code.

HSPA (HSP70) nucleotide binding domain (NBD)

Despite the sequence differences between the HSPA6 and DnaK proteins, their NBD domain tertiary structures are similar (Figures 15A and B). As shown in Figure 15C, the NBD domain of HSPA6 (PDB id: 3FE1) shown in green, and HSPA1A (PDB id: 1HJO) shown in red, exhibited a high level of similarity as shown by structural alignment, with all the lobes, subdomains and turns of the two proteins overlapping each other. Therefore, both the sequence and structures of the NBD domain in HSPA6 and HSPA1A are very similar. These results are consistent with other studies, where functional differences in the HSPA members have been mainly attributed to the SBD domain since a high level of sequence and structural similarity exist in the NBD domain among these members (Wisniewska et al. 2010).

HSPA (HSP70) substrate binding domain (SBD)

Compared to the NBD domain, the crystallization of the SBD domain has proven to be challenging. No SBD structure is available for the HSPA6 and HSPA1A proteins. The structure that is available is a partial C-terminus part of the alpha-helical domain (Table 1). Therefore, the protein structure of the substrate-bound SBD domain of DnaK (Figure 16, PDB id: 1DKZ) (Zhu et al. 1996) was used for the analysis of amino acid differences between the SBD domains of HSPA6 and HSPA1A.

Based on the DnaK protein structure (Figure 16), the amino acids that are in proximity to the bound substrate (shown in yellow) are highlighted in blue. The corresponding amino acids in the
Figure 15. Structure similarity of the nucleotide binding domains of HSPA6, HSPA1A, and the bacterial homologue DnaK proteins

A) Protein structure of the NBD domain in the bacterial homologue DnaK (PDB id: 2KHO). B) Protein structure of the NBD domain of HSPA6 (PDB id: 3FE1). The structures are shown with the same color-coding of the lobes and subdomains which are labeled on HSPA6 structure. C) NBD domains of HSPA6 (green) and HSPA1A (red) are aligned using the PyMol program (www.pymol.org). The bound nucleotides in HSPA6 and HSPA1A structure are shown in purple and blue, respectively.
Due to a lack of a complete SBD structure for HSPA6 and HSPA1A, the corresponding structure of the substrate-bound DnaK protein (PDB id: 1DKZ) was used with the same color-coding of $\beta$-SBD (burgundy) and $\alpha$-helical lid (green) used in previous sections (Figures 12, 13, and 15). The amino acids that are in the proximity of the bound substrate (shown in yellow) in the $\beta$-SBD binding cleft are highlighted in blue. The stick representation of amino acid side chains are shown on the parts of the $\alpha$-helical domain that closes on the $\beta$-SBD, with the amino acids facing the $\beta$-SBD highlighted in purple. Two orientations of the same structure with a 90° rotation are shown.
β-SBD domain are identified in the sequence alignment of HSPA6 and HSPA1A proteins by blue lines above the amino acids (Figure 13). This analysis indicated that, as previously suggested by others (Noonan et al. 2008b), the amino acids potentially involved in substrate binding are identical in HSPA6 and HSPA1A.

The amino acids located on the α-helical lid domain (Figure 16; green) that lock on the β-SBD domain are shown in stick representation with the amino acids facing the β-SBD domain highlighted in purple. The positions of the corresponding amino acids are identified on the sequence alignment of HSPA6 and HSPA1A proteins by 9 purple circles above the amino acids (Figure 13). As observed in Figure 13, these 9 amino acids that face the β-SBD are more conserved than the amino acids directed away from the β-SBD. This indicates that both the amino acids that are potentially involved in substrate binding, and the ones that lock on the substrate binding domain (in the ADP-bound state), are identical in HSPA6 and HSPA1A proteins.

The conserved interdomain linker sequence

The leucine-rich hydrophobic linker sequence is conserved among HSPA (HSP70) family members and comprised of approximately 14 amino acids (shown in cyan in Figures 12, 13) that connects the NBD and SBD domains (Laufen et al. 1999; Mayer et al. 1999; Han and Christen 2001; Vogel et al. 2006). The conformational changes in the NBD domain induced by nucleotide binding or hydrolysis are transferred to the SBD domain by allosteric communication via the linker sequence (da Silva and Borges 2011; Zhuravleva et al. 2012). This linker communication, which is critical for chaperone activity, is bidirectional as the binding of the substrate to the SBD
domain also induces the hydrolysis of the ATP in the NBD domain (Karzai and McMacken 1996; Laufen et al. 1999).

Figure 13 indicates that the linker sequences of HSPA6 and HSPA1A proteins are \textsuperscript{386}KC\textsubscript{E}K\textsubscript{V}Q\textsubscript{D}L\textsubscript{L}L\textsubscript{L}\textsubscript{LD}VA\textsuperscript{399} and \textsuperscript{386}KS\textsubscript{E}N\textsubscript{V}Q\textsubscript{D}L\textsubscript{L}L\textsubscript{L}\textsubscript{LD}VA\textsuperscript{399}, respectively. There are two amino acid differences between these linker sequences; S\textsubscript{387}C and N\textsubscript{389}K (shown in bold letters). The leucine-rich segment which is flanked by an aspartic acid (D) on either side (ie. DLLLLD), is the most conserved and critical segment of this sequence. Mutation of VLLL in the linker domain of bacterial Hsp70 homologue, DnaK, to AAAA or mutation of LL to DD, blocks the allosteric communication of the two domains so that neither binding of a new ATP causes the release of the substrate nor the binding of DnaJ and substrate induces the hydrolysis of ATP to ADP (Laufen et al. 1999). In order to investigate the conservation of this linker sequence, and in particular the two different amino acids, among human HSPA members and that of mouse and rat which lack the HSPA6 gene, the corresponding sequences were extracted from the Ensembl database (www.ensembl.org) and compiled into Table 2.

As observed in Table 2, within the human HSPA members, except for the more variable HSPA5 and HSPA9 sequences that are found in endoplasmic reticulum (ER) and mitochondria, respectively, the other HSPA members (HSPA1A, HSPA1B, HSPA1L, HSPA2, HSPA6, and HSPA8) have a conserved linker sequence. The Lys (K) residue at the fourth position of the linker sequence is only observed in HSPA6 and human and rat HSPA1L that is localized to testis. All the other sequences in human, mouse, and rat, except the variable HSPA5 and HSPA9, have an asparagine (N) in this position.

Interestingly, all sequences in human, mouse, and rat HSPA members, except the variable HSPA5 and HSPA9, contain a serine (S) at the second position of the linker sequence, which is
Table 2. Linker sequence of human, mouse, and rat HSPA (HSP70) family members

Using the Ensembl database (www.ensembl.org) the linker sequence of the human, mouse, and rat HSPA family members were compiled. Those members (e.g. pseudogenes) that showed a high level of sequence dissimilarity such that the linker sequence was unrecognizable were excluded from the analysis. Human HSPA6 sequence is shown in red. The position of the two amino acids in the linker sequence that are different between human HSPA6 and HSPA1A are indicated in all sequences using two transparent boxes.
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<td>ENSRNOp00000058593</td>
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replaced by a cysteine (C) only in the human HSPA6 linker sequence (Table 2). Serine and cysteine are both small amino acids of comparable size, with a hydroxyl (-OH) group in serine being replaced by a thiol (-SH) group in cysteine.

There are eight cysteine amino acids in HSPA6; four in the NBD domain (alignment positions 19, 108, 269, and 308), one in linker domain (position 387), and three in the SBD domain (positions 576, 605, and 624) (see yellow triangles in Figure 13). Five of these cysteines are shared by HSPA1A; three in NBD domain (positions 19, 269, and 308) and two in SBD domain (positions 576 and 605). The other three cysteines in HSPA6 are replaced by serine (position 108 in the NBD domain and 387 in the linker domain) and phenylalanine (position 624 in the SBD domain) in HSPA1A (see yellow triangles with stars in Figure 13). The presence of these additional three cysteines in HSPA6, specially the one at the linker sequence, could potentially result in a higher likelihood of disulfide bond formation in HSPA6 compared to HSPA1A.

In order to assess the proximity of the eight cysteine residues on the HSPA6 protein structure as a measure of their potential for disulfide bond formation, their positions were mapped on to the DnaK reference structure (Figure 17), except for Cys624 which was located beyond the C-terminus of the crystalized DnaK structure. An analysis of the other seven cysteines revealed that Cys269 and Cys308 (in the IIB region of NBD domain) and Cys576 and Cys605 (in the α-helical region of the SBD domain) are close to each other. However, Cys108 (IB region of NBD) and Cys387 (linker sequence) that are only present in HSPA6 (designated by stars in Figures 13 and 17) are not close to any other cysteine residues.

The change from a serine in HSPA1A to a cysteine in HSPA6 in the conserved linker sequence could have two potential consequences on post-translational modification of HSPA6, namely disulfide bond formation and phosphorylation, that will be discussed in the following sections.
Figure 17. Mapping the positions of HSPA6 cysteine residues on the DnaK reference protein structure

The location of HSPA6 cysteine residues are shown with yellow sphere representations on the closed (A) and open (B) conformations of DnaK. The amino acid locations correspond to those shown in the HSPA6 and HSPA1A sequence alignment (Figure 13). The cysteine residues present in HSPA6 but not HSPA1A are designated by a star. For both closed and open conformations two orientations of the same structure are shown.
A

Closed conformation

B

Open conformation
5 DISCUSSION

5.1 Stress-induced localization of HSPA6 and HSPA1A proteins to centrioles in cultured differentiated human neuronal cells

In order to identify potential cytoplasmic stress-sensitive sites in differentiated human neuronal cells, localization of YFP-tagged HSPA6 and HSPA1A proteins following thermal stress was investigated in differentiated SH-SY5Y cells. Immediately following thermal stress, a strong cytoplasmic signal was detected in YFP-HSPA6 and YFP-HSPA1A transfected cell lines, which was not detected in the YFP control cell line. It was speculated that the signal emanated from the centrosome, which was confirmed by employing a centrosome specific marker protein, γ-tubulin. While the YFP-HSPA1A centrosome signal was diminished by the 2 h time point after thermal stress, the YFP-HSPA6 signal continued up to the 3 h time point.

In neurons, centrosomes play key roles in cellular polarity and migration (Tsai and Gleeson 2005; Higginbotham and Gleeson 2007; de Anda et al. 2010; de Anda and Tsai 2011). Aberrations in these structures have been linked to neurodegenerative diseases (Bornens 2002; Badano et al. 2005; Diaz-Corrales et al. 2005; Bradshaw et al. 2008; Diaz-Corrales et al. 2011; Kuijpers and Hoogenraad 2011). Centrosomes are composed of two perpendicular barrel-shaped microtubule-based cylinders termed 'centrioles' surrounded by pericentriolar material (Bornens 2002; Azimzadeh and Bornens 2007; Bettencourt-Dias and Glover 2007; Azimzadeh and Marshall 2010; Nigg and Stearns 2011; Bornens 2012; Brito et al. 2012; Gonczy 2012).

During differentiation, neurons go through a bipolar stage that is critical for their maturation (Nadarajah et al. 2001; LoTurco and Bai 2006; Barnes and Polleux 2009). After treatment with
retinoic acid, cultured SH-SY5Y cells exhibited a bipolar morphology with extended neural cellular processes. In the literature, studies on the localization of HSPA proteins have focused on dividing cells, regarding the centrosome as an entity rather than individual centrioles with proximal and distal ends (Brown et al. 1996; Hut et al. 2005; Scieglnska et al. 2008). In order to study the centriolar localization of YFP-tagged HSPA6 and HSPA1A proteins in more detail, γ-tubulin and centrin antibodies were employed that identify the proximal and distal ends of the centrioles, respectively. It was found that both proteins localize to the proximal end rather than the distal ends of centrioles in differentiated human neurons following thermal stress.

Centrioles are polar structures that exhibit structural and functional differences at their distal and proximal ends. The distal end is involved in microtubule nucleation, whereas, the proximal end has a fibrous network that connects the two centrioles, and also a cartwheel structure that forms the assembly and stabilizing base of the barrel-shaped centrioles (Bornens 2002; Azimzadeh and Bornens 2007; Azimzadeh and Marshall 2010; Bornens 2012). Interestingly, proteins at the proximal end of the centriole have been implicated in the formation of the primary cilium (Molla-Herman et al. 2008), a stress-sensitive antennae-like structure that plays essential roles in the regulation of sensory and signaling systems during neurogenesis (Breunig et al. 2008; Spassky et al. 2008; Baudoin et al. 2012; Prodromou et al. 2012). Dysfunction of the primary cilium leads to neurological disorders, termed 'ciliopathies', that have pronounced effects on neural development (Green and Mykytyn 2010; Lee and Gleeson 2011; Louvi and Grove 2011). Localization of HSPA proteins to the proximal end of the centriole in differentiated human neurons suggests that this cytoplasmic structure is stress-sensitive and that proteins in the cellular stress response may play roles in protecting it against stress-induced damage.
Neurogenesis occurs during early development of the brain but also throughout adult life (Lindsey and Tropepe 2006; Ming and Song 2011; Kempermann 2012; Rojczyk-Golebiewska et al. 2014; Vadodaria and Jessberger 2014). Protection of neurogenesis through HSPA-mediated stabilization of centrioles could be beneficial in the treatment of neurodegenerative diseases, which are characterized by neuronal loss (Mehler and Gokhan 2000; Culmsee and Landshamer 2006). It has been noted that aberrations in centrosomal proteins are linked to brain disorders (Badano et al. 2005; Diaz-Corrales et al. 2005; Bradshaw et al. 2008; Diaz-Corrales et al. 2011; Kuijpers and Hoogenraad 2011).

HSPA6 is present in the human genome, however, it is not found in mouse and rat (Chow and Brown 2007; Noonan et al. 2007b; Noonan et al. 2008b). Hence a component of a centriole defense mechanism in the human brain could be missing in current animal models of human neurodegenerative diseases. Evolution of the very large human brain imposes a high demand on neuronal migration during development (Letinic and Rakic 2001; Rao and Wu 2001). Much greater distances must be traversed in the human brain, compared to the rodent brain, as differentiating neurons migrate to their functional sites in the nervous system. In addition, a pathway for neuronal migration has been reported in the human brain that is not present in other mammals (Letinic and Rakic 2001; Rao and Wu 2001; Clowry et al. 2010). This suggests that neuronal migration has played a key role in the evolution of the human brain and its development.

The presence in human of HSPA6 that rapidly localizes to the proximal end of centrioles following cellular stress, and resides there longer than HSPA1A, could protect neuronal migration from cellular stress in the human brain. HSPA6 was found to exhibit specificity for the client protein p53 (Hageman et al. 2011), which has been shown to localize to the centrosome
A differential phosphorylation of the HSPA6 protein compared to HSPA1A, which will be discussed later in Section 5.3, could also potentially contribute to the longer association of HSPA6 at the proximal end of the centriole, as phosphorylation has been shown to be a regulatory mechanism affecting the assembly, activation, and stabilization of proteins at the centrosome (Reboutier et al. 2012; Conduit et al. 2014; Mbom et al. 2014).

5.2 YFP-tagged HSPA6 and HSPA1A proteins associate with nuclear structures following thermal stress

Another objective of this thesis was the identification of stress-sensitive sites in the nucleus of differentiated human SH-SY5Y neuronal cells. Immediately following thermal stress at the 20 min time point, YFP-HSPA6 and YFP-HSPA1A proteins associated with type I nuclear structures that localized with nuclear speckles. At the 2 h time points, both proteins associated with type II nuclear structures that localized to the GC component of nucleolus. In the recovery period, at the 3 h time point after thermal stress, YFP-HSPA6 protein localized to type III nuclear structures around the periphery of nuclear speckles, which was not observed in YFP-HSPA1A transfected cells.

In the past, the nucleus was viewed as a mass of chromosomal fibers, however, with advances in electron and fluorescence microscopy, the nucleus is now recognized as a highly dynamic and organized structure divided into chromosome territories with multiple nuclear bodies (Dundr and Misteli 2001; Schneider and Grosschedl 2007; Fedorova and Zink 2008). Characterization of
nuclear bodies is an active area of research (Zimber *et al.* 2004; Handwerger and Gall 2006; Matera *et al.* 2009; Mao *et al.* 2011; Meldi and Brickner 2011; Lusic *et al.* 2013) as is the investigation of their alteration in human diseases including neurodegeneration (Donmez-Altuntas *et al.* 2005; Woulfe 2008; Pietrzak *et al.* 2011; Lusic *et al.* 2013). In the following sections, the stress-induced localization of YFP-HSPA6 and YFP-HSPA1A proteins to nuclear structures will be discussed.

5.2.1 YFP-tagged HSPA6 and HSPA1A proteins localize to type I structures at nuclear speckles

The present results show the localization of YFP-HSPA6 and YFP-HSPA1A protein with nuclear speckles immediately after thermal stress. This identifies the nuclear speckles of differentiated human neuronal cells as potential stress-sensitive sites. Nuclear speckles are enriched in RNA splicing factors (Spector and Lamond 2011). More than 80% of the proteins that are localized to nuclear speckles are involved in RNA synthesis and splicing (Mintz *et al.* 1999; Saitoh *et al.* 2004). Following heat stress, major metabolic cellular processes are inhibited in an attempt to conserve energy and enhance cell survival (Velichko *et al.* 2013). Inhibition of mRNA splicing following heat shock has been demonstrated by several groups (Yost and Lindquist 1986; Bond 1988; Utans *et al.* 1992; Shin *et al.* 2004; Biamonti and Caceres 2009) and can occur as a result of degradation or modification of factors involved in the formation of spliceosome (Utans *et al.* 1992; Shin *et al.* 2004). The absence of intron sequences in stress-inducible heat shock genes enables them to avoid disruption of mRNA splicing due to thermal stress.
It is known that following stress-induced splicing arrest, mRNA molecules accumulate in nuclear speckles (Johnson et al. 2000; Melcak et al. 2000) and that Hsps contribute to the splicing recovery process (Yost and Lindquist 1991; Corell and Gross 1992; Marin-Vinader et al. 2006). For example, Hsp27 has been shown to speed up the recovery of RNA splicing in heat shocked cells by facilitating the recognition of the pre-mRNA 5’ splice sites by the spliceosome (Marin-Vinader et al. 2006). Similarly, the localization of YFP-HSPA6 and YFP-HSPA1A proteins to nuclear speckles may facilitate the recovery of the splicing activity following heat stress.

5.2.2 YFP-tagged HSPA6 and HSPA1A proteins associate with type II nuclear structures in the granular component (GC) of nucleolus

At the 2 h time point after heat shock, YFP-HSPA6 and YFP-HSPA1A proteins associated with type II nuclear structures that localized to the outermost granular component (GC) of the nucleolus which is involved in rRNA processing and ribosomal subunit assembly (Raska et al. 2006; Sirri et al. 2008; Hernandez-Verdun et al. 2010). These results identify the GC component of nucleolus as another nuclear stress-sensitive site in differentiated human neuronal cells.

The nucleolus is the cellular centre of ribosome biogenesis and houses a variety of proteins that are involved in cell cycle control and DNA replication (Boisvert et al. 2007). It also plays a fundamental role in sensing and responding to cellular stress and has been termed the ‘hub of the stress response’ (Boulon et al. 2010). It has been suggested that dysfunction of the nucleolus may contribute to the onset of neurodegenerative diseases (Hetman and Pietrzak 2012).

The localization of YFP-tagged HSPA6 and HSPA1A proteins to the GC component of the nucleolus could enhance the recovery of the heat-sensitive rRNA processing and ribosomal
subunit assembly. This could in turn facilitate the formation of ribosomes that are required for the resumption of protein synthesis following heat-induced translation inhibition (Parag et al. 1987; Lam et al. 2007). Meeting the high demand for the ribosomes is a vital cellular task. Abnormalities in ribosome biogenesis have been linked to human diseases such as treacher collins syndrome, male infertility, and anauxetic dysplasia (Freed et al. 2010), and there has been increasing interest in targeting the nucleolus in therapeutic approaches (Pickard and Bierbach 2013).

The localization of Hsps to the nucleolus of heat stressed cells has been reported by several groups (Pelham 1984; Welch and Feramisco 1984; Milarski et al. 1989; Kotoglou et al. 2009). Binding of Hsp70 to damaged ribosomal RNPs leads to their reassembly and improves the recovery of nucleolar morphology following heat shock (Pelham 1984; Velichko et al. 2013). Protection of ribosome biogenesis by Hsp70 has been observed in several species including E. coli (Maki et al. 2002; Rene and Alix 2011), yeast (Albanese et al. 2010) and plant cells (Nover et al. 1986). Electron microscopy has demonstrated that Hsp70 localizes to the GC component of the nucleolus of tomato cells following heat shock, which promotes pre-ribosome formation (Nover et al. 1986). The association of Hsp70 with the GC component of the nucleolus has been viewed as a ribosomal step in the translational recovery process after thermal inhibition (Welch and Feramisco 1984; Welch and Mizzen 1988; Beck and De Maio 1994; Knowlton and Salfity 1996). The present results suggest that the HSPA6 and HSPA1A proteins may both be involved in the recovery of rRNA processing and ribosome assembly following cellular stress.
5.2.3 YFP-HSPA6 protein associates with type III nuclear structures at the periphery of nuclear speckles

Later in the recovery period at the 3, 5, and 7 h time points, YFP-HSPA6 protein associated with type III nuclear structures that localized at the periphery of nuclear speckles. This phenomenon was not observed in YFP-HSPA1A transfected cells. The periphery of nuclear speckles, also known as ‘perispeckles’ (Schmidt et al. 2006; Daguenet et al. 2012; Li et al. 2013), is site of active mRNA transcription (Wei et al. 1999; Spector and Lamond 2011) that is enriched in RNA polymerase II (Bregman et al. 1995; Mortillaro et al. 1996) and poly(A)-containing RNA (Hall et al. 2006). The majority of transcribed mRNAs also undergo co-transcriptional splicing in the periphery of nuclear speckles (Girard et al. 2012).

The effect of the transcription inhibitor, α-amanitin, on the localization of type III structures around the periphery of nuclear speckles in YFP-HSPA6 transfected cells was investigated. This drug blocks the activity of RNA polymerase II (Lindell et al. 1970; Nguyen et al. 1996) and results in the enlargement and rounding of nuclear speckles (Kumaran et al. 2002; Tabellini et al. 2003; Xie et al. 2006). The present results demonstrated that following α-amanitin treatment, type III nuclear structures localized to the periphery of enlarged nuclear speckles at the 3 h time point after heat shock. This suggested that these HSPA6 positive nuclear structures are tethered at the periphery of nuclear speckles.

Enrichment of perispeckles in poly(A)-containing RNAs and RNA polymerase II, suggested that localization of type III nuclear structures is related to the synthesis and/or processing of RNA molecules. To investigate this, YFP-HSPA6 transfected cells were treated with either RNase or DNase. The results indicated that type III nuclear structures that were present in YFP-HSPA6 transfected cells at the 3 h time point after heat shock, were sensitive to RNase but not DNase.
digestion. These two experiments suggested that localization of YFP-HSPA6 protein to type III nuclear structures is RNA dependent.

Thermal stress results in the inhibition of mRNA transcription (Allen et al. 2004; Hieda et al. 2005; Espinoza et al. 2007; Mariner et al. 2008; Mitchell and Fraser 2008; Yakovchuk et al. 2009; Velichko et al. 2013) and splicing (Yost and Lindquist 1986; Bond 1988; Utans et al. 1992; Shin et al. 2004; Biamonti and Caceres 2009). Association of YFP-tagged HSPA6 protein with type III structures that localize to the periphery of nuclear speckles (perispeckles) that are sites of active transcription and splicing (Wei et al. 1999; Spector and Lamond 2011; Girard et al. 2012), suggest that HSPA6 may interact with transcription and/or splicing machinery following thermal stress. In addition to facilitating resumption of RNA synthesis and processing, HSPA6 may stabilize nascent pre-mRNA at sites of active transcription. For example, HSPA6 may be involved in preventing self-hybridization of pre-mRNAs that contain long introns prior to splicing. Increasing the stability of mRNAs through binding has been demonstrated for Hsp70, Hsp110 (Henics et al. 1999) and Hsp90 (Wang et al. 2011).

Species that possess the HSPA6 gene could benefit from a potentially faster resumption of RNA transcription and/or splicing following stress-induced inhibition, which would be critical in the human brain that engages in higher cognitive functions compared to mouse and rat. The HSPA6 gene is found in human genome and not in mouse and rat, hence current animal models of neurodegenerative diseases are lacking a potentially neuroprotective protein (Chow and Brown 2007; Noonan et al. 2007b; Noonan et al. 2008b; Khalouei et al. 2014).

Overall, the results presented in this thesis identify the proximal end of centrioles, nuclear speckles and the outer GC component of nucleolus as potential stress-sensitive sites in differentiated human neuronal cells given their association with HSPA6 and HSPA1A proteins.
Interestingly, HSPA6, but not HSPA1A, localized to the periphery of nuclear speckles at time points when transcription and RNA splicing is recovering from cellular stress. Hence, HSPA6, that is present in the human genome but not in rat and mouse, may be involved in nuclear homeostasis in differentiated human neurons. Nuclear speckles and the nucleolus are the principle nuclear components that are involved in mRNA transcription/splicing and ribosome biogenesis (Cisterna and Biggiogera 2010; Spector and Lamond 2011). Disruption in transcriptional regulation and RNA splicing contributes to neurodegenerative diseases (Cha 2000; Riley and Orr 2006; Nelson and Keller 2007; Bithell et al. 2009; Ricciardi et al. 2009; Caldeira et al. 2013) as does nucleolar malfunctions such as aberrations in ribosomal RNA synthesis and ribosome biogenesis (Boisvert et al. 2007; Nelson and Keller 2007; Freed et al. 2010; Pietrzak et al. 2011; Parlato and Kreiner 2013).

5.3 **Sequence and structural analysis of YFP-HSPA6 and YFP-HSPA1A proteins**

A comparison analysis of the sequence and structure of HSPA6 and HSPA1A proteins was performed. Protein structures of the nucleotide binding domain (NBD) of these proteins have been crystalized and are publicly available (Table 1). The present analysis showed that the sequence and structure of the NBD domains of HSPA6 and HSPA1A proteins are similar. Compared to the NBD domain, the substrate binding domain (SBD) contains more sequence differences between the two proteins. Since there is no protein structure available for the complete SBD domains of HSPA6 and HSPA1A proteins, the bacterial homologue DnaK was
employed. Using this reference structure, it was found that the amino acids at the substrate binding site and at the alpha-helical lid domain that lock on the substrate, are conserved between the HSPA6 and HSPA1A proteins.

The HSPA6 and HSPA1A protein sequences share five cysteine (C) residues. The HSPA6 sequence contains an additional three cysteine residues. Two of these result from the replacement of serine residues in the HSPA1A sequence by cysteine residues in the HSPA6 sequence at amino acid positions 108 and 387 (located in the NBD domain and the linker sequence, respectively) (Figure 18). Replacement of a phenylalanine (F) amino acid in the HSPA1A sequence by a cysteine in the HSPA6 sequence at position 624 in the SBD domain, accounts for the third additional cysteine residue in the HSPA6 protein sequence (Figure 18). The Ser387 residue in the highly conserved linker sequence of the HSPA1A protein is shared among other HSPA members (HSPA1A, HSPA1B, HSPA1L, HSPA2, and HSPA8). HSPA6 is the only protein in this family that exhibits the Ser387Cys substitution (see Table 2).

Cys108 and Cys387 are exposed on the surface in the open conformation of the protein (based on the analysis of the DnaK reference structure) and could potentially affect disulfide bond formation and phosphorylation of HSPA6 protein compared to HSPA1A (Earnhart et al. 2011; Nishi et al. 2011). The position of Cys624 in HSPA6 sequence cannot be mapped on the DnaK reference structure since the last segment (about 30 amino acids) of the alpha-helical SBD domain of the DnaK protein is missing in the crystalized structure.
Figure 18. Positions of the three additional cysteine residues in HSPA6 protein

The nucleotide binding domain (NBD), substrate binding domain (SBD) and the conserved linker sequence (boxed area) connecting these domains are shown in the schematic diagram of the HSPA protein members. The approximate positions of the three additional cysteine residues in HSPA6 protein, namely Cys108 in the NBD domain, Cys387 in the linker sequence, and Cys624 in the SBD domain are designated with a star (*). These star-designated Ser108Cys, Ser387Cys, and Phe624Cys amino acid substitutions, along with the Asn389Lys (ie. N389K) in the linker sequence of HSPA6, are shown with bold letters in the corresponding sequence alignment boxes.
Potential effect of the cysteine substitutions on HSPA6 protein disulfide bond formation

Cysteine residues are the only amino acids that can participate in disulfide bond formation, a covalent bond that stabilizes protein structures (Tu and Weissman 2004). Interestingly, both Cys108 and Cys387, that are found in HSPA6 but not HSPA1A, are exposed to the surrounding environment (in ‘open’ protein conformation; Figure 17B) which increases the likelihood of inter-molecular disulfide bond formation that can potentially result in protein oligomerization (Earnhart et al. 2011; Bellmann et al. 2014). This is particularly important in stressed neuronal cells that can undergo alterations in their oxidation-reduction status, which in turn can affect the balance of disulfide bond formation. Interestingly, following exposure to oxidant species, some HSPA family members exhibit increased disulfide-linked conformers, while other members show a decreased level (Cumming et al. 2004). Therefore, the presence of the two exposed cysteine residues (Cys108 and Cys387) in HSPA6 protein that are absent in HSPA1A protein, could increase the potential of disulfide bond formation in HSPA6, potentially affecting its self-oligomerization or binding to other proteins. Inducible members of the HSPA family have been shown to form disulfide bonds with other cytoplasmic proteins in a mammalian neuronal cell line (Cumming et al. 2004). The extra three cysteine residues in the HSPA6 protein may influence its function. It has been suggested that redox sensing by specific cysteine residues in the HSPA1A protein, that are not present in the constitutive HSPA8 protein, may contribute to the responses to oxidative stress (Miyata et al. 2012).

Oligomerization of Hsp70 has been shown to involve interaction of the linker sequence and the SBD domain (Aprile et al. 2013). In another study Cys576 in the SBD domain of this protein has been implicated in dimer formation (Nemoto et al. 2006). The extra cysteine residues in the
NBD, linker sequence, and SBD domain, may make the HSPA6 protein more prone to oligomerization compared to HSPA1A. The potential effects of oligomerization on the localization of HSPA6 protein to type III nuclear structures at the periphery of nuclear speckles remains to be investigated.

The three substitutions from serine and phenylalanine in HSPA1A to cysteine in HSPA6 are interesting considering the low frequency of cysteine usage in protein sequences in general, and particularly in HSPA6 and HSPA1A, where cysteine constitutes 1% of the total amino acid composition. Frequency of cysteine residues in molecular chaperones has been shown to be much lower than other protein families (Kumarevel et al. 1998; Fu et al. 2003).

5.3.2 Potential effect of the serine to cysteine substitution on HSPA6 protein phosphorylation

Serine residues are the most frequently phosphorylated amino acids in proteins, followed by threonine and tyrosine (Pearlman et al. 2011). Phosphorylation is known to play a role in functional regulation of proteins resulting in either inhibition or activation of protein activity (Cohen 2000). More than 30% of eukaryotic cellular proteins become phosphorylated (Ubersax and Ferrell 2007; Holt et al. 2009), a process that has been shown to have direct effects on human health and disease (Cohen 2001). Phosphorylation has also been shown to affect the cellular localization of Hsps such as Hsp27 (Schmidt et al. 2012). Interestingly, the two additional cysteine residues in HSPA6 (Cys108 and Cys387), that are exposed to the surrounding environment (see previous section) are both substituted from a serine residue in HSPA1A (Figure 18). These two serine to cysteine substitutions could potentially lead to less
phosphorylation of HSPA6 protein compared to HSPA1A which could result in inhibitory or activation effects on HSPA6 protein.

Similar to the aforementioned disulfide bond formation, phosphorylation has also been implicated in protein-protein binding with phosphorylated residues usually located on binding interfaces (Nishi et al. 2011). Certain protein domains recognize phosphorylated amino acids on other proteins and bind to them. Manipulation of these events has been considered in cancer therapeutic approaches (Watanabe and Osada 2012). Phosphorylation affects the intranuclear localization of factors involved in mRNA splicing (Stamm 2008). Through differential phosphorylation, HSPA6 could interact with a different set of proteins compared to HSPA1A, which could in turn contribute to its localization to type III nuclear structures located around the periphery of nuclear speckles.

Overall, the results of sequence and structural analysis of HSPA6 and HSPA1A proteins, suggested that their NBD domain is more conserved than the SBD domain. The additional exposed cysteine residues in the HSPA6 sequence, namely Cys108 in the NBD domain, Cys387 in the linker sequence, and possibly Cys624 in the SBD domain, could potentially contribute to differential features of the HSPA6 protein compared to HSPA1A via disulfide bond formation and phosphorylation that results in its stress-induced localization to type III nuclear structures.

An interesting future experiment is site-directed mutagenesis of the two exposed cysteine residues, and in particular Cys387 in the linker sequence of HSPA6, converting it to a serine residue to see if such a mutation causes the disappearance of type III nuclear structures. Alternatively, the exposed serine residues in HSPA1A (Ser108 in the NBD domain and Ser387
in the linker sequence) could be mutated to cysteine residues to study the effect of these mutations on the appearance of type III nuclear structures. These experiments would provide support for the involvement of these cysteine substitutions, in the NBD domain and the linker sequence, on the localization of YFP-HSPA6 protein to type III nuclear structures at the periphery of nuclear speckles.

5.4 Conclusion

Compared to other widely studied members of the HSPA family (Kiang and Tsokos 1998; Evans et al. 2010; Young 2010), little attention has been given to HSPA6. Two inducible members of the HSPA family, HSPA6 and HSPA1A, share 84% sequence identity which raises the question of whether they perform similar or different cellular functions. One way to address this is to investigate their intracellular localization following stress. In this thesis, stressed-induced localization of YFP-tagged HSPA6 and HSPA1A proteins to cytoplasmic and nuclear structures was investigated in order to identify potential stress-sensitive sites in differentiated human neuronal cells and explore similarities and differences in the localization pattern of these two proteins in differentiated SH-SY5Y human neuronal cells following cellular stress.

YFP-HSPA6 and YFP-HSPA1A proteins rapidly localized to the proximal end of centrioles, with the former protein exhibiting a longer localization following thermal stress. Both proteins also localized to nuclear speckles (type I structures), followed by their localization to the GC component of nucleolus (type II structures). Later in the recovery period, YFP-HSPA6 localized to the periphery of nuclear speckles (type III structures), which was not observed in YFP-HSPA1A transfected cells.
These results identified the proximal end of the centrioles, nuclear speckles, and the GC component of nucleolus as potential stress-sensitive sites in differentiated human neuronal cells. HSPA6 and HSPA1A proteins may be involved in the stabilization of centrioles, recovery of RNA splicing, and ribosome biogenesis following thermal stress. Localization of the YFP-HSPA6 protein to perispeckles suggests that this site is stress-sensitive in differentiated human neuronal cells, and indicate a unique feature of this little studied member of the HSPA family in the recovery of transcription and/or splicing following cellular stress. The longer association of HSPA6 protein at the proximal end of the centrioles compared to HSPA1A, and the localization of HSPA6 to perispeckles, highlight differences between these two inducible members of the HSPA family in both the cytoplasm and the nucleus.

The gene coding for HSPA6 protein is present in the human genome but not in the genomes of rat and mouse, hence current animal models of neurodegenerative diseases are missing this potentially protective heat shock protein. In addition to exhibiting similar localization with HSPA1A to centrioles, nuclear speckles and the GC component of nucleolus, HSPA6 may facilitate recovery of RNA transcription and splicing following stress-induced inhibition in differentiated neuronal cells.

The results of the sequence and structural analysis of HSPA6 and HSPA1A proteins, revealed three additional cysteine residues in HSPA6 that are located in the nucleotide binding domain (Cys108), the linker sequence (Cys387), and the substrate binding domain (Cys624). These three cysteine residues could contribute to differential features of the HSPA6 protein compared to HSPA1A through disulfide bond formation and/or phosphorylation. Interestingly, the cysteine in the linker sequence of HSPA6 is not found in any other members of the HSPA family.
6 REFERENCES


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# Appendix: one- and three-letter codes of amino acids

<table>
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<th>One Letter Code</th>
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