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ORIGINAL PAPER

Targeting of Heat Shock Protein HSPA6 (HSP70B′) to the Periphery of Nuclear Speckles is Disrupted by a Transcription Inhibitor Following Thermal Stress in Human Neuronal Cells

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Abstract Heat shock proteins (Hsps) are a set of highly conserved proteins involved in cellular repair and protective mechanisms. The intracellular localization of inducible members of the HSPA (HSP70) family can be used as an index to identify stress-sensitive sites in differentiated human neuronal cells. Following thermal stress, the little studied HSPA6 (HSP70B′) was targeted to the periphery of nuclear speckles (perispeckles) that are sites of transcription factories. Triptolide, a fast-acting transcription inhibitor, knocked down levels of the large subunit of RNA polymerase II, RPB1, during the time-frame when HSPA6 associated with perispeckles. Administration of triptolide to heat shocked human neuronal SH-SY5Y cells, disrupted HSPA6 localization to perispeckles, suggesting the involvement of HSPA6 in transcriptional recovery after stress. The HSPA6 gene is present in the human genome but is not found in the genomes of the mouse and rat. Hence current animal models of neurodegenerative diseases lack a member of the HSPA family that exhibits the feature of stress-induced targeting to perispeckles.

Keywords HSPA6 (HSP70B′) · Human neuronal SH-SY5Y cells · Triptolide

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Introduction

Following exposure to a range of stressful stimuli, cells exhibit a highly conserved heat shock (stress) response during which protein production is inhibited and a set of heat shock proteins (Hsps) is induced [1–3]. Hsps repair stress-induced protein damage and protect cells against future stress [4, 5]. Stress-inducible Hsp members are expressed in response to physiological and environmental stimuli to combat protein misfolding and aggregation [5–9]. Up-regulation of Hsps has been proposed as a potential therapeutic strategy for neurodegenerative diseases, which have been characterized as protein misfolding disorders [10–18]. Hsps represent a line of defense against misfolded, aggregation-prone proteins [13, 16, 19].

The HSPA (HSP70) is a multi-gene family with stress-inducible HSPA1A (HSP70-1) being the most abundant and widely studied member [20–32]. Comparatively little attention has been given to stress-inducible HSPA6 (HSP70B′) which is present in the human genome, and not found in the genomes of mouse and rat [33–38]. Hence current animal models of neurodegenerative diseases lack a member of the HSPA family that is present in the human genome. At present, few effective therapies for human neurodegenerative diseases have been identified despite numerous clinical trials [19, 39, 40]. Therapeutic compounds that have been identified and appeared promising in animal models of neurodegenerative diseases, have repeatedly failed to translate to effective treatments in human clinical settings. This has led to concerns about deficiencies in current animal models of human neurodegenerative diseases.

Following thermal stress, HSPA6 localized to nuclear speckles, which are enriched in RNA splicing factors (identified by the marker protein SC35), and to the granular component of the nucleolus that is involved in rRNA processing

and ribosomal subunit assembly (identified by the marker protein nucleophosmin) [41–47]. HSPA6 also localized to the periphery of nuclear speckles ('perispeckles') that are sites of transcription factories [48–52]. The present study investigated the effect of triptolide, a fast-acting transcriptional inhibitor that is effective at nanomolar concentrations [53]. Results demonstrated that triptolide disrupted the heat-induced targeting of HSPA6 to perispeckles, however the localization of HSPA6 to nuclear speckles and the nucleolus was not affected. Current mouse and rat models of neurodegeneration, that lack HSPA6, are missing a feature of the heat shock response exhibited by human neuronal cells that is associated with transcription recovery after stress.

Materials and Methods

Growth of Human Neuronal SH-SY5Y Cells

Human neuronal SH-SY5Y cells stably expressing YFP-tagged HSPA6 (HSP70B′) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), and cultured at 37 °C in a humidified 5% CO_2 atmosphere. Plasmid preparation, transfection, and selection of SH-SY5Y cells constitutively expressing eYFP-HSPA6 were carried out as previously described [54]. Transfected cells were plated in 10 cm dishes at 5×10^6 cells per cm^2 for Western blotting, and onto glass coverslips at 5×10^4 cells per cm^2 for immunocytochemistry. Differentiation of neuronal cells was induced with 10 μM all-*trans*-retinoic acid under serum-free conditions at 37 °C for 72 h [55].

Western Blotting

Differentiated human neuronal SH-SY5Y cells were harvested and solubilized in Laemmli buffer and boiled for 15 min. Protein quantitation was carried out using the RC DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal loadings of 50 μg of protein per lane were separated by 7% SDS-PAGE and 4% stacking gel, using the Mini-PROTEAN 3 Electrophoresis Module Assembly (Bio-Rad Laboratories) before transfer to nitrocellulose membranes. Western blotting was carried out with primary antibodies specific for RPB1 (ab140509, Abcam, Cambridge, UK), and β-tubulin (MAB3408, EMD Millipore, Billerica, MA, USA) as loading control. Secondary antibodies, peroxidase-conjugated donkey anti-goat (Jackson ImmunoResearch Labs, West Grove, PA, USA) and goat anti-mouse (A4416, Sigma Aldrich, St. Louis, MO, USA), were detected using enhanced chemiluminescence (Luminata™ Classico Western HRP Substrate, EMD Millipore). Western blots representative of three experimental repeats are shown.

Heat Shock Treatment

Differentiated human neuronal SH-SY5Y cells transfected with YFP-HSPA6 were incubated under control conditions (37 °C) or heat shocked at 43 ± 0.1 °C for 20 min in a circulating water bath and then transferred to a recovery incubator at 37 °C with 5% CO_2 for 20 min. Cells were treated with the indicated nanomolar concentration of triptolide dissolved in DMSO in serum-free DMEM.

Immunocytochemistry

YFP-HSPA6 transfected human neuronal SH-SY5Y cells were harvested at the indicated time points, with time zero being the commencement of heat shock at 43 °C. Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4) at room temperature for 30 min and then permeabilized with 0.1% Triton-X 100 and 100 mM glycine in PBS for 30 min. After washing with PBS, cells were blocked with 5% fetal bovine serum (FBS) in PBS for 1 h, followed by incubation with primary antibodies in 1% FBS in PBS overnight at 4 °C. Primary antibodies specific for nuclear speckles (SC35, ab11826, Abcam) and the granular component of the nucleolus (nucleophosmin, ab37659, Abcam) were used. Cells were subsequently washed and incubated with fluorescently labeled cy3 and cy5 donkey anti-mouse and donkey anti-rabbit secondary antibodies for 2 h at room temperature (Alexa Fluor®, Thermo Fisher Scientific, MA, USA). The cells were mounted and viewed using a Quorum Wave FX-X1 spinning disc confocal system (Quorum Technologies, Ontario, Canada) using a 63X oil objective. Images were captured via a Hamamatsu electron-multiplying charge-coupled device camera. For Fig. 3b, 100 cells were sampled, and the average counts of three independent experiments were used for statistical analysis. Data was expressed as the mean ± the standard deviation of the means. An unpaired Student's *t* test of unequal variance was used to assess significant differences ($*p < 0.05$).

Results

The Transcriptional Inhibitor Triptolide is Effective at Nanomolar Concentrations

Triptolide has recently been characterized as a fast-acting transcriptional inhibitor [53, 56]. It induces the hyper-phosphorylation of the carboxyl-terminal domain of the RPB1 subunit of RNA polymerase II, which targets it to the proteasome for degradation [57–59]. Differentiated human

neuronal SH-SY5Y cells stably transfected with YFP-HSPA6 were treated with triptolide at nanomolar concentrations ranging from 50 to 500 nM. As demonstrated by Western blotting in Fig. 1a, 100 nM triptolide reduced levels of RPB1, the large subunit of RNA polymerase II. The time course of the reduction of RPB1 by 100 nM triptolide is shown in Fig. 1b. These observations led to the selection of 100 nM triptolide for the investigation of the effect of this transcriptional inhibitor on localization of YFP-HSPA6 to stress-sensitive sites in differentiated human neuronal cells.

Localization of YFP-HSPA6 to Nuclear Structures Following Thermal Stress in Differentiated Human Neuronal Cells

YFP-tagged HSPA6 localized to nuclear components following heat shock and recovery at 37 °C, as shown in Fig. 2. YFP-tagged HSPA6 associated with nuclear speckles, which were identified using the nuclear speckle marker protein SC35 (indicated by arrowheads in Fig. 2) [60, 61]. Nuclear speckles are enriched with splicing factors that are involved in the processing of mRNA [46]. YFP-HSPA6 also localized to the granular component of the nucleolus, identified using the marker protein nucleophosmin (indicated by double arrowheads in Fig. 2) [45]. The granular component is engaged in ribosomal RNA processing and ribosomal subunit assembly [42, 44, 45].

As shown in Fig. 2, in the magnified areas of the lower two panel sets, YFP-tagged HSPA6 was subsequently targeted to perispeckles located at the periphery of nuclear speckles which have been characterized as sites of transcription factories (perispeckles, indicated by arrows clustered around SC35-positive nuclear speckles which are identified by arrowheads) [48–52].

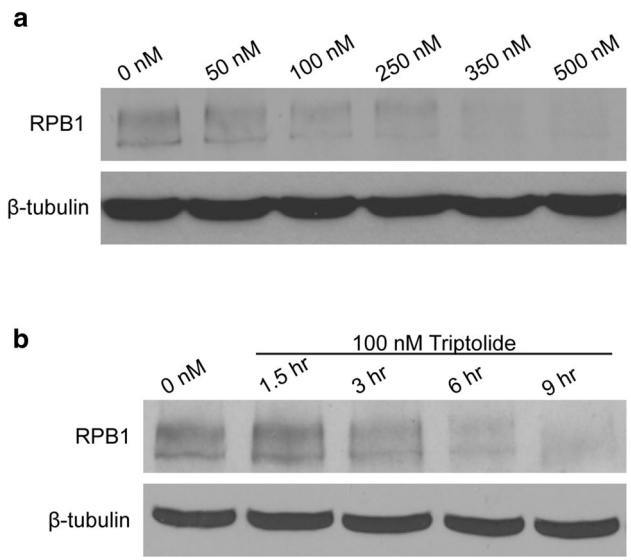

Fig. 1 Nanomolar concentrations of triptolide decreased levels of RPB1, the large subunit of RNA polymerase II, in differentiated human neuronal SH-SY5Y cells. **a** Western blot showing the effect of 50–500 nM triptolide on RPB1 levels following 3 h of incubation. **b** Time course of the effect of 100 nM triptolide on RPB1. β-tubulin was employed as the loading control

Triptolide Disrupted the Targeting of YFP-HSPA6 to Perispeckles in Human Neuronal Cells

The localization of HSPA6 to perispeckles, that have been characterized as sites of transcription factories, suggested that HSPA6 may be involved in the recovery of transcription following stress in human neuronal cells. As shown in Fig. 3a, triptolide disrupted the targeting of YFP-tagged HSPA6 to the periphery of nuclear speckles (perispeckles). YFP-tagged HSPA6 localized to perispeckles in heat-stressed neuronal cells (indicated by arrows in the upper panels of Fig. 3a). However, treatment with triptolide disrupted this association (indicated by the lack of arrows in the lower panels of Fig. 3a at 3 and 6 h). Localization of YFP-HSPA6 to nuclear speckles and the granular component of the nucleolus were not affected (indicated by single and double arrowheads, respectively).

Disruption of the localization of YFP-HSPA6 to the periphery of nuclear speckles during the recovery period from thermal stress was confirmed as shown in Fig. 3b. In the absence of triptolide, 66 ± 1 % of cells demonstrated YFP-HSPA6 targeting to perispeckles, however only 4 ± 1 % of cells demonstrated YFP-HSPA6 localization in the triptolide condition at 3 h. The percentage of cells positive for YFP-HSPA6 at perispeckles was 63 ± 1 % (no triptolide) and 2 ± 1 % (triptolide condition) at 6 h. These results demonstrated that nanomolar concentrations of the transcription inhibitor, triptolide, disrupted YFP-tagged HSPA6 localization at the periphery of nuclear speckles. To determine whether the association of YFP-HSPA6 with perispeckles could be restored following triptolide treatment, triptolide was washed out of cells at 3 h and replaced with serum-free media. Washout of triptolide at 3 h after heat shock did not restore localization of YFP-tagged HSPA6 to the periphery of nuclear speckles (Fig. 3c).

Discussion

Available models for the study of neurodegenerative disease range from cell culture and brain slice systems to whole animals. Cell culture allows the study of complex relations between molecular events and cellular physiology [62]. Human neuronal SH-SY5Y cells have been used as a model to study neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease [63–68]. SH-SY5Y cells can be differentiated to produce neuronal-like phenotypes

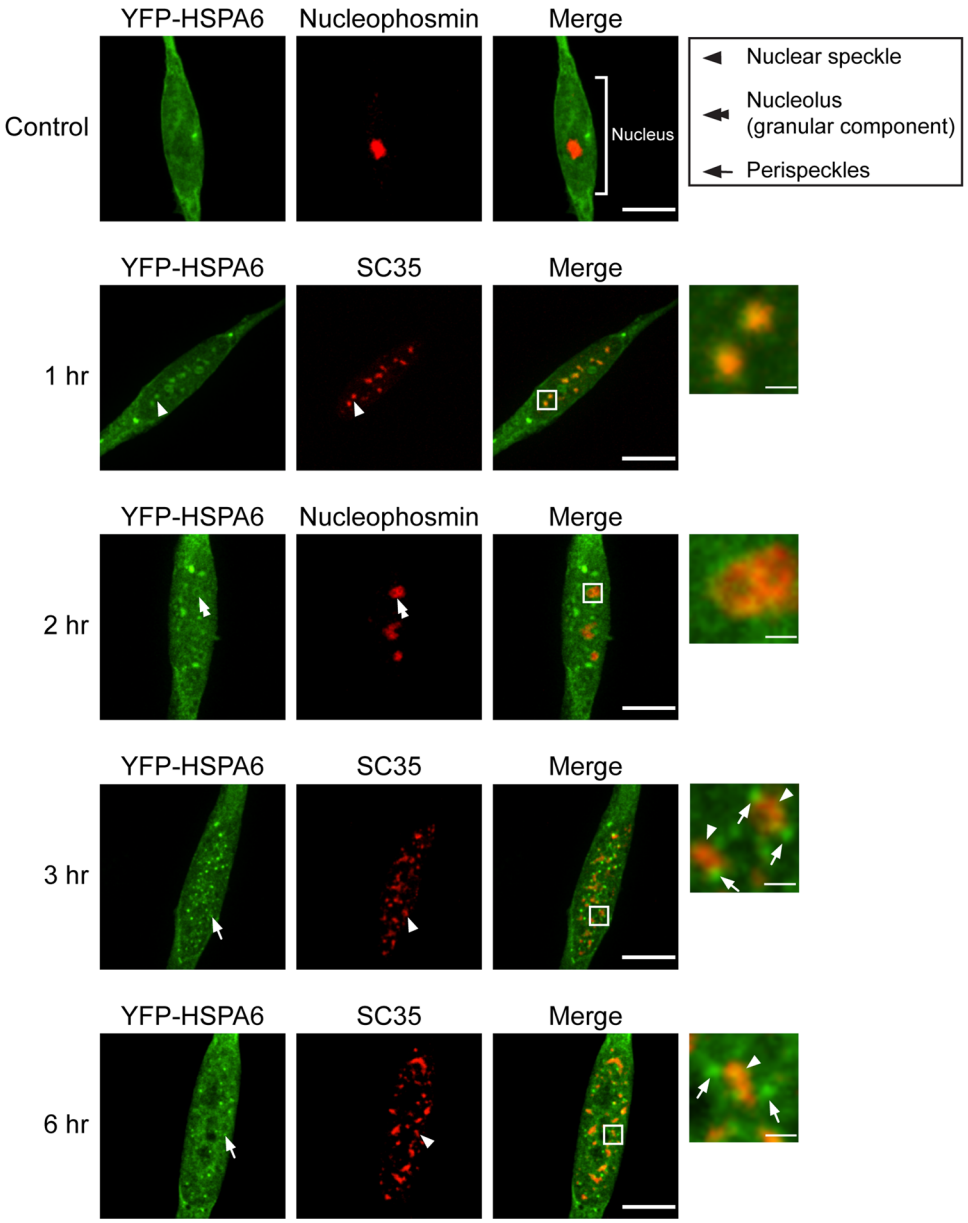

Fig. 2 Association of YFP-tagged HSPA6 protein with nuclear structures in differentiated human neuronal cells following thermal stress. YFP-HSPA6 co-localized with SC35, a marker of nuclear speckles (*arrowhead*), and with nucleophosmin, which identified the granular component of the nucleolus (*double arrowhead*). Subsequently, targeting was observed of YFP-tagged HSPA6 to perispeckles located at the periphery of nuclear speckles (*arrows* perispeckles; *arrowheads* SC35-positive nuclear speckles). The *boxed areas* in *merged panels* correspond to magnified areas that are shown to the *right of each panel*. Time in hour equals time after the start of heat shock. *Scale bars* equal 10 μm (*panel images*), and 1 μm (*magnified insets*)

[66, 69–71]. The present study employed retinoic acid, which is an endogenous signaling molecule for neuronal differentiation during in vivo development of the nervous system [72–74]. SH-SY5Y cells, differentiated with retinoic acid, develop long bipolar "neuronal-like" processes [75] and express biochemical markers of neuronal differentiation [62, 63, 66, 68, 76, 77]. Since the HSPA6 gene is present in the human genome, SH-SY5Y neuronal cells are an appropriate model to investigate this little studied member of the HSPA family, which is not present in mouse and rat models of neurodegenerative diseases, in order to explore whether HSPA6 exhibits features not observed for the widely studied HSPA1A. This could reveal features of the cellular stress response that are lacking in current animal models of neurodegenerative diseases due to the absence of HSPA6, which is strictly inducible and does not exhibit detectable levels of endogenous, basal expression in unstressed cells [33, 38].

The targeting of Hsps to specific intracellular structures identifies stress-sensitive sites in neuronal cells and suggests that Hsps facilitate recovery processes following exposure to stress. Nuclear speckles are enriched in RNA splicing factors [41, 43, 46]. In an attempt to conserve energy and enhance survival, cellular metabolism is down regulated following thermal stress [3]. RNA splicing is inhibited following exposure to heat shock [78–81]. Introns are absent from stress-inducible heat shock genes, hence they are not affected by stress-induced inhibition of RNA splicing. Hsps contribute to the recovery of the splicing process [78, 82, 83]. This is accomplished through facilitating the recognition of pre-mRNA 5′ splice sites by the splicesome [83]. The

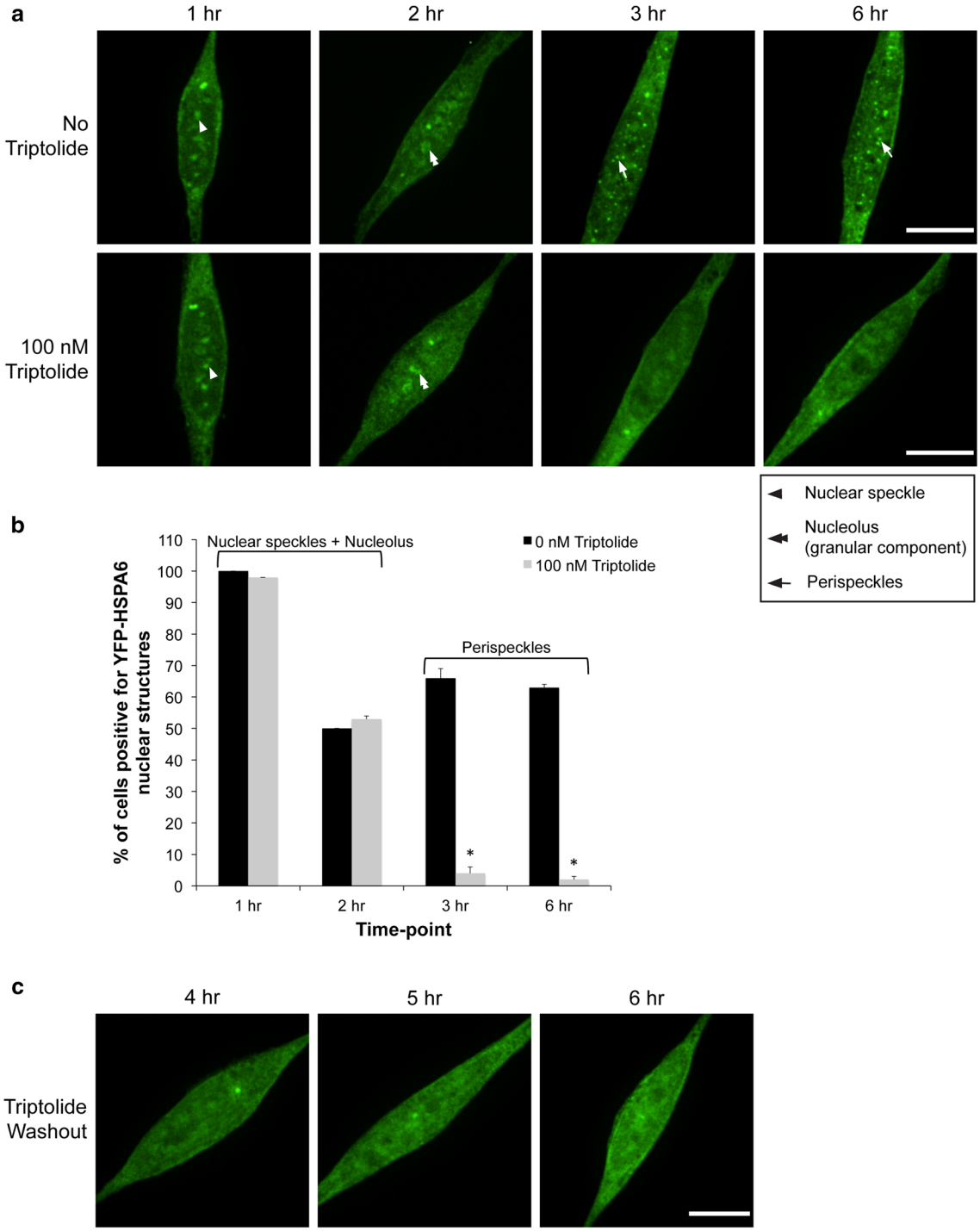

Fig. 3 Triptolide disrupted the targeting of YFP-tagged HSPA6 to perispeckles. **a** *Upper panel* following exposure to thermal stress, YFP-tagged HSPA6 localized to nuclear speckles (*arrowheads*), the granular component of the nucleolus (*double arrowheads*), and subsequently the periphery of nuclear speckles (perispeckles, indicated by *arrows*). *Lower panel* application of 100 nM triptolide disrupted heat-induced YFP-HSPA6 targeting to perispeckles (indicated by the lack of *arrows* at 3 and 6 h). **b** Cells positive for YFP-HSPA6 localization to nuclear structures, with or without triptolide treatment. YFP-tagged HSPA6 was targeted to perispeckles in 66 % ± 1 % of cells at 3 h, and 63 % ± 1 % at 6 h. However, following triptolide treatment, only 4 ± 1 % and 2 ± 1 % of heat shocked cells demonstrated YFP-HSPA6 localization to perispeckles at 3 and 6 h, respectively (*$p < 0.05$). Data were expressed as the mean ± standard deviation of the means. **c** Washout of triptolide at 3 h did not restore the localization of YFP-HSPA6 to perispeckles at 4, 5, and 6 h. Time in hour equals time after the start of heat shock. *Scale bars* equal 10 μm

localization of YFP-HSPA6 to nuclear speckles in human neuronal SH-SY5Y cells following heat shock suggests that HSPA6 plays a role in splicing recovery.

HSPA6 also localized to the granular component of the nucleolus, which is the site of ribosomal RNA processing and ribosomal subunit assembly [42, 44, 45]. This could contribute to the recovery of ribosome biogenesis, which is required to resume protein synthesis following exposure to thermal stress [84, 85]. The nucleolus is a hub of the stress response because of its role in sensing and responding to cellular stress [86]. Neurodegenerative diseases have been linked to nucleolar dysfunction, and the nucleolus has been targeted for therapeutic approaches [87, 88]. This highlights the potentially protective role of HSPA6 in the nucleolus. It has been reported that triptolide does not decrease levels of the RPA135 and RPA194 subunits of RNA polymerase I [57]. Ribosomal RNA is transcribed by RNA polymerase I primarily at the border of the fibrillar center and the dense fibrillar component of the nucleolus [89], not the granular component that is the site of HSPA6 localization. This may account for the observation that triptolide does not disrupt the localization of HSPA6 to the granular component of the nucleolus.

HSPA6 is targeted by thermal stress to perispeckles, which have been characterized as sites of transcription factories enriched in transcription factors [48–52]. Nanomolar concentrations of triptolide, a fast-acting transcription inhibitor [53, 56], disrupted the stress-induced localization of HSPA6 to perispeckles suggesting that HSPA6 is associated with transcriptional recovery after stress. Interestingly, the widely studied HSPA1A does not localize to perispeckles after thermal stress, however it does localize to nuclear speckles and the nucleolus [47]. This suggests that current mouse and rat models of neurodegeneration that lack HSPA6 are missing a feature of the heat shock response in human neuronal cells that is associated with transcriptional recovery after stress.

Triptolide has recently been identified as a fast-acting transcription inhibitor [53, 56]. Transcription inhibition is induced by the hyper-phosphorylation of the carboxyl-terminal domain of RPB1 of RNA polymerase II, which targets it to the proteasome for degradation [57–59]. The characterization of triptolide as a fast-acting transcriptional inhibitor is particularly relevant to the development of therapies for cancers that advance quickly and aggressively, such as pancreatic cancer and neuroblastoma [90, 91]. Triptolide has recently been reported to inhibit tumor growth and metastases in vivo [92], and hence demonstrates promise as an anticancer drug. However, given the present results, its impact on the heat shock (stress) response in human neuronal cells should be considered.

The localization of HSPA6 to perispeckles in differentiated human neuronal cells, which are characterized as sites of transcription factories [48–52], indicates that these critical nuclear structures are stress-sensitive. The present study demonstrates that administration of triptolide disrupted the targeting of HSPA6 to perispeckles, suggesting that HSPA6 may be associated with transcriptional recovery in differentiated human neuronal cells after cellular stress. Our ongoing experiments indicate that knockdown of HSPA6 sensitizes differentiated human neuronal SH-SY5Y cells to heat stress. It has been reported that HSPA6 knockdown also sensitizes human colon cancer cells to heat shock [34]. The HSPA6 gene is found in humans, and not in the mouse and rat [33, 37, 38]. Hence, current animal models of neurodegenerative diseases lack a member of the HSPA family that exhibits the feature of stress-induced targeting to perispeckles. We have recently compared the dynamics of the association of HSPA6 and HSPA1A with nuclear structures in differentiated human neuronal cells using fluorescence recovery after photobleaching (FRAP) [93]. The stress-induced association of HSPA6 with perispeckles displayed the greatest dynamism compared to the interaction of HSPA6 or HSPA1A with other stress-sensitive nuclear structures [93]. The presence in HSPA6 in the human genome could provide differentiated neuronal cells with a highly dynamic mechanism for transcriptional recovery after stressful stimuli.

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