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HSPB7 is a SC35 speckle resident small heat shock protein

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ABSTRACT

Background: The HSPB family is one of the more diverse families within the group of HSP families. Some members have chaperone-like activities and/or play a role in cytoskeletal stabilization. Some members also show a dynamic, stress-induced translocation to SC35 splicing speckles. If and how these features are interrelated and if they are shared by all members are yet unknown. **Methods:** Tissue expression data and interaction and co-regulated gene expression data of the human HSPB members was analyzed using bioinformatics. Using a gene expression library, sub-cellular distribution of the diverse members was analyzed by confocal microscopy. Chaperone activity was measured using a cellular luciferase refolding assay. **Results:** Online databases did not accurately predict the sub-cellular distribution of all the HSPB members. A novel and non-predicted finding was that HSPB7 constitutively localized to SC35 splicing speckles, driven by its N-terminus. Unlike HSPB1 and HSPB5, that chaperoned heat unfolded substrates and kept them folding competent, HSPB7 did not support refolding. **Conclusion:** Our data suggest a non-chaperone-like role of HSPB7 at SC35 speckles. **General significance:** The functional divergence between HSPB members seems larger than previously expected and also includes non-canonical members lacking classical chaperone-like functions.

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1. Introduction

The human small heat shock protein (small HSP, HSPB) family of chaperones contains a total of eleven family members [33,80]. All HSPB proteins are characterized by a conserved crystallin domain flanked by a variable sized N- or C-terminus resulting in a molecular size of approximately 16–40 kDa. Another well-described characteristic is their ability to oligomerize into large, spherical and symmetrical structures [30]. The main feature of many small HSPs is their ability to interact with components of the cytoskeleton [21,50,59,71,79] and to protect the cytoskeleton during stress [46]. In cells, HSPB1 has been found to affect cell motility and morphology, related to its interaction with cytoskeletal elements [67]. Furthermore, HSPB1 seems to fulfill a protective roles in heart diseases like ischemia [23,83] and atrial fibrillation [7] preventing contractile elements against becoming dysfunctional.

One other well-defined molecular function of HSPB proteins is their ability to prevent aggregate formation of denatured proteins *in vitro* [3,39]. *In vivo*, this basic function is part of the cellular pathways accommodating protein folding and degradation [9,13]. Active processing of HSPB bound substrates occurs in collaboration with HSPA (HSP70) members which can lead to either the refolding or degradation of the substrate. [9,80].

The vital role of HSPB members as chaperones and/or protectors of the cytoskeleton is also apparent from several genetically inherited diseases. Mutation in both HSPB1 and HSPB8 has been reported to be causative in the development of Charcot–Marie–Tooth disease and distal hereditary motor neuropathy [22,37]. Mutated HSPB4 and HSPB5 can both cause cataract [16,49] while desmin-related myopathy is caused by specific mutations in HSPB5 [82].

Another feature of certain HSPB proteins is their involvement in RNA splicing: e.g. HSPB1 up-regulation was found to enhance the recovery of splicing after heat stress [52] which may relate to early findings of enhanced recovery of translation arrest after stress when HSPB1 was overexpressed [48]. This has been associated with findings that, upon a combination of stress and (stress-induced) phosphorylation, HSPB1 associates with nuclear splicing speckles, (or SC35 speckles) [8], which are nuclear domains involved in RNA splicing. Also HSPB5 has been reported to associate with these nuclear speckles in a phosphorylation dependent manner [18]. Interestingly, this HSPB member was shown to recruit the F-box protein FBX4 to the speckles, suggesting a role for HSPB5 in facilitating ubiquitination of speckle components [18,20]. We also found that heat-denatured substrates associate with these nuclear speckles together with HSPB1 and indirect evidence indicated that this was not associated with refolding of denatured substrates [10]. Rather, a hypothetical model was proposed that these nuclear splicing speckles would be used during stress for temporal storage of unfolded proteins to target them for degradation upon stress relief. This model was supported by findings that these speckles largely overlapped with sites for protein degradation [68].

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Whether cytoskeleton-related functions, splicing-related functions and chaperone activity are distinct features of the various HSPB proteins or whether they all are phenotypical manifestations of a single conserved function remains unclear. Also, to what extent the various HSPB members differ in these functional aspects remains to be elucidated. Therefore, we initiated a systematic study on the HSPB1–HSPB10 members of the human HSPB family (HSPB11 was not investigated as it was discovered [4] after we had completed our studies). In this MS, we provide the first part of these data, starting with a general overview, mainly based on bioinformatics, on the tissue-specific expression, intracellular localization patterns, interaction partners and co-regulated genes of the HSPB1–10 members. Using a human HSPB plasmid library, we initially focused on the sub-cellular localization patterns of the HSPB family. Interestingly, we show that one of the least investigated HSPB members, HSPB7, is a SC35-speckle-associated protein under non-stressful conditions. Association of HSPB7 with nuclear speckles is dependent on the N-terminus of HSPB7. While HSPB1 can assist in the refolding of heat-denatured proteins, HSPB7 is ineffective in doing so. Moreover, targeting HSPB1 to the nuclear speckles using the HSPB7 speckle localization signal abolishes its refolding enhancing capacity, strongly suggesting that association with speckles is not associated with chaperone-assisted refolding.

2. Materials and methods

2.1. Reagents and antibodies

Tetracycline, MOPS and cycloheximide were obtained from Sigma (Sigma, Zwijndrecht, The Netherlands), beetle luciferin from Promega (Promega Benelux, Leiden, The Netherlands). Antibodies against the V5 tag (Invitrogen, Breda, The Netherlands) and SC35 (Abcam, Cambridge, United Kingdom) were mouse monoclonal. The antibody against the MYC tag (Abcam, Cambridge, United Kingdom) was rabbit-polyclonal.

2.1.1. Molecular techniques

Standard recombinant DNA techniques were carried out essentially as described by Sambrook et al. [70]. Oligonucleotide primers (Biologio, Nijmegen, The Netherlands) and plasmids used in this study are listed in Tables S1 and S2 respectively. Restriction enzymes were used according to the manufacturer's instructions (Invitrogen, Breda, The Netherlands, New England Biolabs, Ipswich, MA). Preparative polymerase chain reactions (PCR) were carried out using Vent DNA polymerase (New England Biolabs, Ipswich, MA) according to the manufacturer's instructions. DNA sequencing reactions were carried out by ServiceXS (ServiceXS, Leiden, The Netherlands). HSPB protein sequences were retrieved from the NCBI database and analyzed by ClustalX [47] and visualized using Treeview [66].

2.1.2. HSPB plasmid generation

The human HSPB family members were isolated from both mixed cell line cDNA and commercially available cDNA clones (Open Biosystems, Huntsville, USA) (Table S1). PCR products were cloned into the pCDNA5/FRT/TO-MV plasmid (Invitrogen, Breda, The Netherlands) (Fig. S1) and sequence verified. Modification of the pCDNA5/FRT/TO plasmid was performed by cloning of oligonucleotides. This vector is designed to allow, in combination with a tetracycline repressor, for tetracycline-regulated expression (Fig. S1). The HSPB encoding cDNAs were subsequently sub-cloned in frame with either a HIS tag, V5 tag, EGFP, MYC or mRFP tag (for availability see Fig. S1). As such, the resulting library is tailored to a large set of research topics. Deletion of the N-terminus of HSPB7 was performed using the Quickchange kit (Stratagene; Bio-Connect, Huissen, The Netherlands). Generation of chimeric constructs was performed as described before [81].

2.1.3. Growth conditions and DNA transfections

Flp-In T-Rex HEK293 cells expressing the Tet repressor (Invitrogen, Breda, The Netherlands) and HeLa cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (Griener Bio-one, Alphen aan den Rijn, The Netherlands) at 37 °C under a humidified atmosphere containing 5% CO₂. Mouse HL-1 atrial cardiomyocytes were maintained in Claycomb Medium (Sigma, Zwijndrecht, The Netherlands) supplemented with 100 μM norepinephrine (Sigma, Zwijndrecht, The Netherlands) dissolved in 0.3 mM L-ascorbic acid (Sigma, Zwijndrecht, The Netherlands), 4 mM L-glutamine (Gibco; Invitrogen, Breda, The Netherlands) and 10% fetal calf serum (Griener Bio-one, Alphen aan den Rijn, The Netherlands) at 37 °C under a humidified atmosphere containing 5% CO₂. HEK293 cells and mouse HL-1 cells were transfected using Lipofectamine (Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions using 1 μg of plasmid DNA per 35-mm dish. HeLa cells were transfected using Effectene (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions using 0.6 μg of plasmid DNA per 35-mm dish. Gene expression in Flp-In T-Rex HEK293 cells was induced with a final concentration of 1 μg/mL tetracycline.

2.1.4. Microscopy

For microscopy, cells were plated 24 h before transfection. For fixation, the coverslips were washed with cold PBS and fixed for 15 min with methanol (–20 °C). Cells were permeabilized in blocking solution (100 mM glycine, 3% BSA, 0.1% triton) for 1 h followed by 1 h incubation with the primary antibody (V5 anti-mouse, 1:200, SC35 anti-mouse, 1:10,000, MYC anti-rabbit 1:200). After three washing steps, coverslips were incubated for 1 h with CY3-conjugated anti-rabbit secondary antibody (Amersham Biosciences, Little Chalfont, United Kingdom) at 1:200 dilution or with FITC-conjugated anti-mouse secondary antibody (Jackson, Suffolk, United Kingdom) at 1:200 dilution. After three washing steps, the coverslips were mounted using Citifluor mounting medium (Citifluor Ltd, London, United Kingdom).

Images were obtained using an inverted confocal laser scanning microscope (TCS SP2, DM RXE, Leica, Rijswijk, The Netherlands) with a 63×/1.32 NA oil objective. The mRFP_{ruby} fusion protein [24,60] was excited with a 543-nm helium–neon laser line and emission was recorded between 571 and 626 nm using a DD 488/543 dichroic filter.

2.1.5. Luciferase refolding assay

Chaperone activity of HSPB members was assessed by using the luciferase refolding assay [55]. Briefly, HEK293 cells were co-transfected with nuclear targeted luciferase (Nuc-superluc-EGFP) [29] together with HSPB encoding plasmids (1:9 ratio). Two hours after transfection, expression was induced by addition of tetracycline. After 24 h, cells were resuspended and divided into 1 mL portions in tissue-grade 10 mL tubes. The following day, cells were given a heat shock (30 min at 43 °C) in the presence of cycloheximide (20 μg/mL) and 4-morpholinepropanesulfonic acid (MOPS; 20 mM, pH 7.0) in order to inhibit protein synthesis and increase the buffer capacity of the medium respectively. After heat-treatment, cells were allowed to recover before luciferase activity was determined (3 h at 37 °C). Luciferase activity measurements were performed using a Berthold Lumat 9510 luminometer (Berthold Technologies, Vilvoorde, Belgium).

3. Results

3.1. Bioinformatical analysis

Compared to other HSP families, small HSPs show a rather large sequence divergence, in particular in both the N- and C-terminal

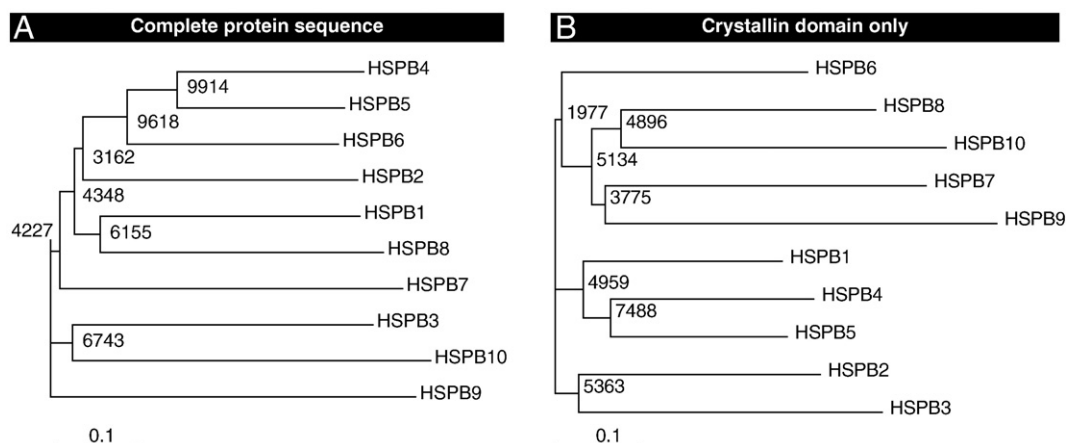


Fig. 1. Nearest neighbor analysis of the human HSPB family. Nearest neighbor analysis of the complete protein sequence of the HSPB members (A) or the crystallin domains only (B). Protein alignments were performed in ClustalX using the Neighbor-joining algorithm in combination with Blosum scoring. Bootstrap values are indicated on the branchpoints. (Bootstrap trails: 10,000.)

regions. A nearest neighbor analysis using the full sequences (Fig. 1A) in order to link functional properties of the diverse member will thus likely be prone to wrong interpretations. And although the two functionally related and interactive members HSPB4 and HSPB5 [36] cluster together in such an analysis (Fig. 1A), HSPB1 does not despite the fact that it shares many functional features with HSPB4 and HSPB5, amongst others of chaperone activity [39]. Rather, in such an analysis HspB1 clusters together with the non-functionally related HSPB8. Therefore, we repeated the nearest neighbor analysis only using the conserved crystallin domains (Fig. 1B). Now, HSPB1 did group together with its functionally related family members HSPB4 and HSPB5. In addition, HSPB2 and HSPB3, which are reported to form functional hetero-oligomers during muscle differentiation [76], now also appear as a (separate) group within the tree, suggesting that this nearest neighbor analysis based on the crystalline domain only might be more informative for function. If correct, this nearest neighbor analysis would then suggest a possible functional overlap between HSPB8/HSPB10 and HSPB7/HSPB9 (Fig. 1B). We have indeed observed a functional overlap between HSPB7 and HSPB9 in handling aggregated substrates (data submitted elsewhere). A functional overlap between HSPB8 and HSPB10 remains to be tested experimentally.

Next, we extracted data from online databases to analyze both interaction data [6] and co-expression data [64] to provide information on interaction partners and co-regulated genes. HSPB members show an extensive set of interaction partners including other HSPB members (Fig. 2B). When looking at gene-networks, HSPB members show co-regulated expression with numerous genes, again including other HSPB genes (Fig. 2A). Clearly these data are yet far from complete as many HSB members have only been poorly studied. Moreover, part of the data only come from cell free studies and yet have to be verified in living cells. But, as can be derived from Fig. 2B, there is a likely complex and extensive interactive network within the HSPB family suggesting that they cooperate in several processes.

In addition to differences in functionality, individual HSPB members show large differences in tissue-specific expression (Fig. 3). HSPB4 for instance, is found at high concentrations in the eye lens, while its expression is almost absent in other tissues [65]. Compiling data from the UniGene EST ProfileViewer (NCBI) (Fig. 3A) for total messenger levels reveals that in the human body as a whole, the most abundant HSPB member is HSPB1, followed by HSPB5 and interestingly also HSPB7 (Fig. 3A top row). Although reported to be expressed almost exclusively in heart tissue [44], messenger RNA for HSPB7 can also be found in adipose tissue, connective tissue and the parathyroid gland. HSPB9, that clusters together with HSPB7 in the nearest neighbor tree (Fig. 1B) has been referred to as a testis specific

HSPB member [42]. But, HSPB9 expression is also found in lung and pancreas tissue.

When comparing HSPB messenger composition for a selection of organs, HSPB1 and HSPB5 represent the most abundant HSPB messengers in brain, muscle and eye tissue (Fig. 3B). Looking at heart tissue, HSPB7 is indeed transcribed at very high levels in comparison to the other HSPB members (Fig. 3B). HSPB messenger composition in the testis shows that HSPB7 and HSPB1 are the most abundant in this organ and not HSPB9. The most restricted members in view of tissue-specific expression are HSPB4 (eye), HSPB9 (lung, pancreas, testis) and HSPB10 (testis) (Fig. 3A).

3.2. Subcellular localization

The small HSPs are known to be widely distributed throughout eukaryotic cellular compartments. Herein, plants take a special place, with the most widely known organelle specific distribution pattern [73]. In *Drosophila* at least one small HSP, the mitochondrial HSP22, shows compartment specific localization besides the cytosol and nucleus [58]. As a complete picture of HSPB protein localization is currently unavailable, we combined web-based prediction programs and subcellular localization databases together with confocal microscopy imaging of the V5-tagged HSPB library to generate a complete overview. *In silico* prediction of subcellular localization was performed using PSORT, Ptarget and Multiloc [27,34,35] (Fig. 4A). There was a surprisingly large disagreement between the predictions made by these different programs especially with regard to a tentative mitochondrial localization signal. Using MITOPRED [28], a web server dedicated specifically for the prediction of mitochondrial localized proteins, HSPB5 and HSPB7 were predicted to be mitochondrial with high confidence, while the other members were not predicted to localize to mitochondria. For HSPB5, a mitochondrial localization was indeed recently experimentally verified [40]. However, neither the LOCATE database [74] nor the Human Protein Reference (HPR) database [56] indicated that the mitochondrial localization of HSPB5 and large differences in the predicted localization and experimentally verified localizations were seen, especially when using the Ptarget server.

To investigate and directly compare the localization of HSPB proteins experimentally, we expressed the V5-tagged HSPB members in human HEK-293 cells (Fig. 4B). Endogenous HSPB1, the most extensively investigated HSPB member is known to be mostly cytosolic under non-stress conditions and only enters the nucleus upon stress-induced phosphorylation and changes in oligomeric size after which it co-localizes with SC35 positive nuclear speckles [8,9]. Using MCF-7 cells that express high levels of HSPB1, endogenous

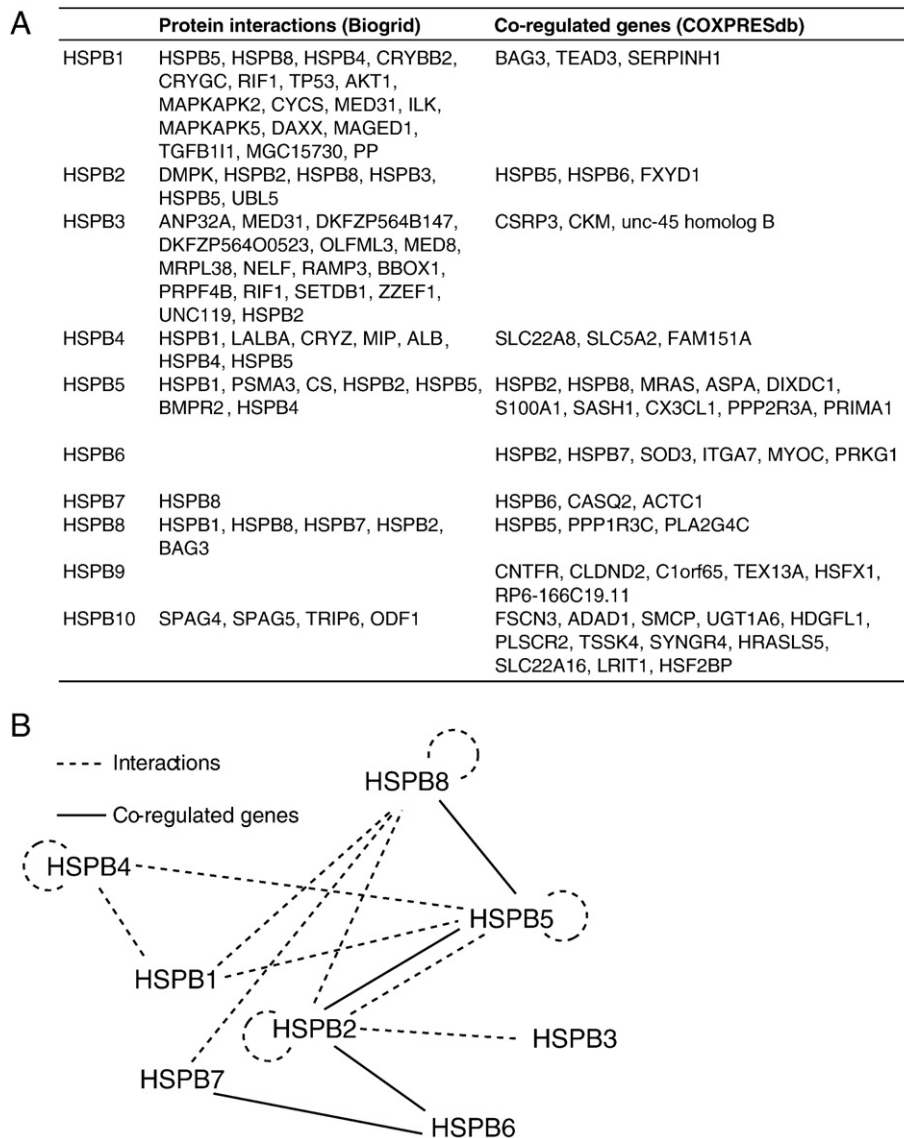


Fig. 2. Physical interactions and co-regulated gene expression of the HSPB family members with other proteins/genes. (A) Database searches were performed to collect information on HSPB interactions with other proteins and co-regulated genes. (B) An overview on both physical interactions (dotted line) and co-regulated genes (straight line) within the HSPB family.

HSPB1 indeed showed mainly cytoplasmic staining in unstressed cells and a heat-induced localization to nuclear speckles (Fig. 4C). Staining of the ectopically expressed V5-HSPB1 in HEK293 cells, showed a similar pattern of expression during control and heat-shocked conditions (Fig. 4D), showing that the V5-tag did not interfere with its physiological localization patterns. Also several experimental data are available for HSPB4 and HSPB5. Like HSPB1, HSPB4 was reported to be localized in the cytoplasm [2,51]. A similar pattern is seen for the ectopically expressed V5-tagged HSPB4 (Fig. 4B). HSPB5 has been reported to be a cytosolic and nuclear protein where it can specifically localize to SC35 positive nuclear speckles [62,78] in a phosphorylation dependent manner [18,19]. Again, the transiently expressed V5-tagged HSPB5 confirmed the cytosolic staining representative for non-phosphorylated HSPB5. Also a faint signal of nuclear foci could be detected, confirming that like untagged HSPB5, also V5-HSPB5 in part associates with nuclear domains. The findings on these three well-studied and closely related HSPB members (Fig. 1B) show that their cellular compartmentalization is highly comparable and also show that V5-tagging does not interfere with their intracellular localization. Also, V5-tagging of HSPB1, HSPB4 or HSPB5 did not affect their function as chaperone in

cells to support refolding of heat-denatured luciferase (Fig. 8A). Apparently for these members tagging does not affect biological activity.

The V5-tagged HSPB2 was found to be present in the nucleus and cytoplasm, consistent with the data from LOCATE. In addition, HSPB2 was present in foci throughout both cellular compartments. HSPB2 has been reported previously to localize to mitochondria [61], we were however unable to detect overlap of the cytosolic HSPB2 foci and the mitochondrial marker Cox8-dsRED (data not shown) suggesting either errors in the predictive programs or a inhibitory effect of the V5 tag on mitochondrial import. The nature of the nuclear foci was not elucidated. To date, no experimental data are available on the localization of HSPB3, the protein that is most closely related to HSPB2 (Fig. 1B). Unlike HSPB2, however, but consistent with the database predictions, V5-HSPB3 showed mainly a cytosolic localization with some minor nuclear staining. No clear localization to specific other organelles or sub-nuclear domains was seen.

Also for the intracellular localization of HSPB6 experimental data are lacking. The V5-HSPB6 confirmed the predicted cytosolic distribution but could not confirm the mitochondrial localization as predicted by Ptarget.



Fig. 3. HSPB mRNA levels throughout the human body. (A) The top row indicates the amount of total mRNA present in the human body for each HSPB member, relative to HSPB1. The vertical view shows the distribution in mRNA expression for each HSPB member in individual organs relative to the organ with the highest HSPB mRNA abundance. (B) Comparison of HSP1, HSPB5, HSPB7 and HSPB9 mRNA expression in either brain, heart, muscle, eye or testis. Values for gene expression (transcript per million) were retrieved from UniGene (NCBI) and calculated relative to the most abundant HSPB member. These values were transformed into grayscale values (0% = white, 100% = black) using Adobe Photoshop.

HSPB8 has been reported to be an exclusively cytosolic protein [14] although others also have suggested that it is localized to the nucleus in some cells [37]. The ectopically expressed V5-HSPB8 shows a predominant cytosolic staining, with some intense staining in cytosolic foci. These foci could potentially represent autophagosomes, consistent with the suggested function of HSPB8 in autophagy [12]. Its closest homologue HSPB10, known to be a structural protein in sperm tails [25], also mainly localized to the cytosol with minor nuclear staining. The expression was however accompanied by the appearance of many cytoplasmic foci and the formation of an aggresome, suggesting misfolding and/or failed clearance of the ectopically expressed V5-tagged protein [41].

The V5-tagged HSPB9 showed a diffuse cytosolic and nuclear distribution (Fig. 3B), whereas *in vivo* HSPB9 shows mainly a nuclear localization in testis and a cytosolic/nuclear distribution in tumor cells [17]. Again this localization was not predicted correctly by the *in*

silico prediction programs (Fig. 3A). Staining of HSPB7 showed a rather striking distribution. HSPB7 was reported to interact with α -Filamin, an actin-binding protein [44], suggesting a cytoskeletal localization. The V5-HSPB7 showed cytosolic staining (Fig. 3B). In addition, however, bright nuclear foci could be detected, that suggested association with nuclear sub-domains in the absence of stress.

During stress, several heat shock proteins are known to be re-allocated within the cells and especially move to the nucleus, including to nuclear speckles [8,26,31,77]. Therefore, we analyzed heat-induced alterations in intracellular localization for all the individual HSPB members. Interestingly, most members appeared after heat shock in nuclear foci or showed an enhanced nuclear foci signal in comparison to control conditions (Fig. S2a). HSPB-related foci, however, did not change in size and intensity. The clearest change in subcellular distribution was found for HSPB1 and HSPB4, which

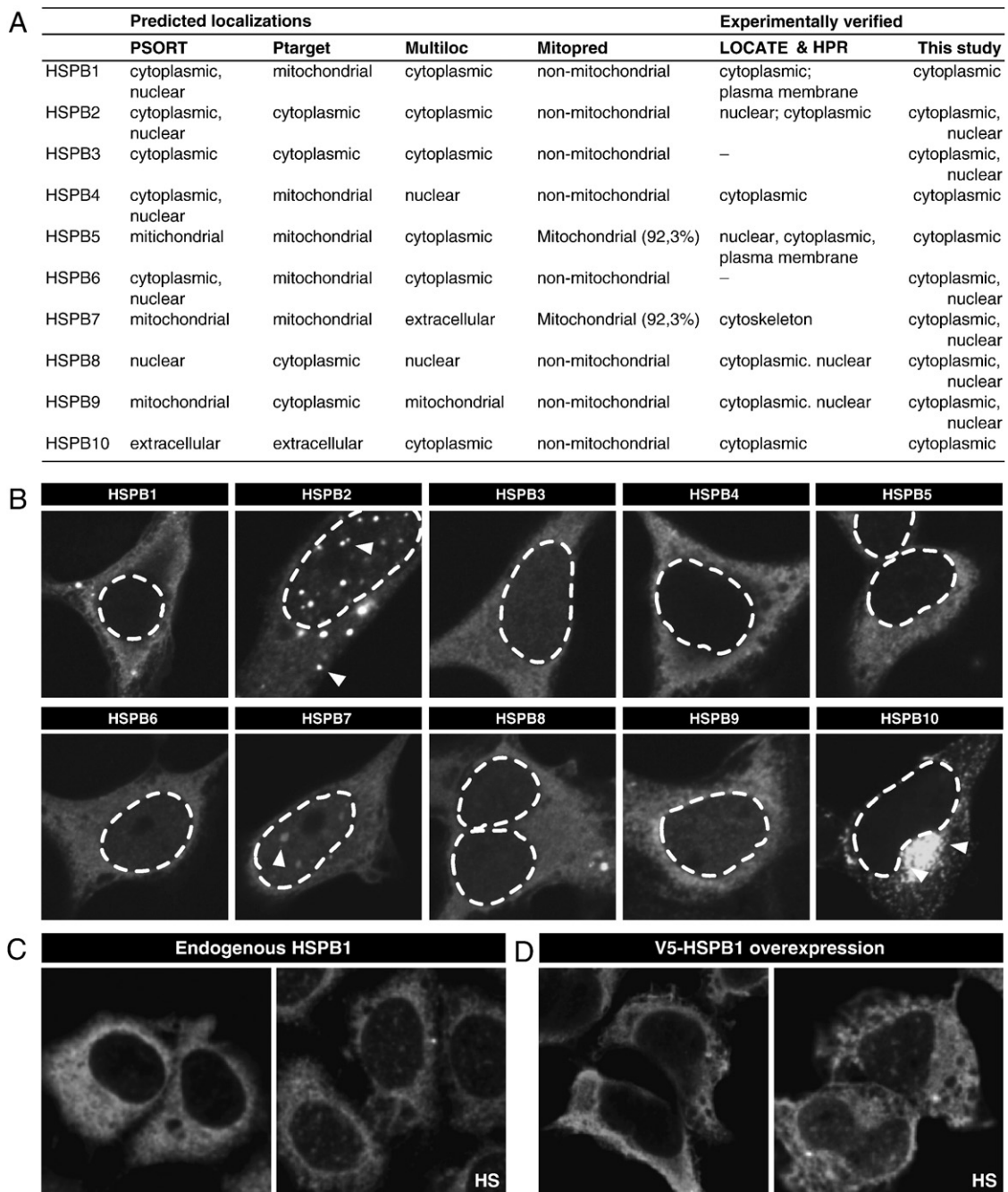


Fig. 4. Intracellular distribution of the human HSPB. (A) Several databases were used to extract the predicted intracellular distribution and published intracellular distribution. (B) Intracellular distribution of V5-tagged human HSPB proteins in HEK293 cells. Arrows indicate bright foci in both the nucleus and the cytoplasm (V5-HSPB2), nuclear foci (V5-HSPB7) and aggresome formation (V5-HSPB10). The nucleus was visualized by expression of the human Histone-2B fused to the monomeric red fluorescent protein ruby [60,69]. This signal was traced in Adobe Photoshop and depicted as a dashed line. (C) Redistribution of endogenous HSPB1 to nuclear speckles after a heat shock. (D) Redistribution of exogenous V5-HSPB1 to nuclear speckles after a heat shock.

both entered the nucleus after heat shock treatment and appeared at nuclear foci (Fig. S2a, b).

3.3. HSPB7 specifically associates with Cajal bodies and SC35 speckles

Because of the association of HSPB1 with splicing and the stress-induced reallocations of HSPB1 and HSPB5 to SC35 splicing speckles, we decided to identify to which specific nuclear structure(s) HSPB7 localizes. Hereto, we co-expressed different nuclear sub-domain markers tagged with EGFP or EYFP with MYC tagged HSPB7. EGFP-tagged SP100 was used to visualize promyelocytic leukaemia (PML) nuclear bodies which play a role in transcriptional regulation,

programmed cell death, tumor suppression, and antiviral defense [5,43]. These structures were in close proximity to MYC-HSPB7-containing bodies, but did not overlap (Fig. 5A). EGFP-tagged Coilin, marking the Cajal bodies, implied in spliceosomal snRNP assembly and various other functions [75] showed partial overlap with the MYC-HSPB7 signal (Fig. 5B), indicating that the HSPB7 can localize to Cajal bodies. Splicing speckles were visualized using SC35 and ASF/SF2; two members of the serine/arginine (SR) rich protein family. These proteins form a group of structurally and functionally related splicing factors which are essential for constitutive and alternative splicing regulation in higher eukaryotes [11,57]. Expression of EGFP-tagged alternative splicing factor/splicing factor 2 (ASF/SF2) showed

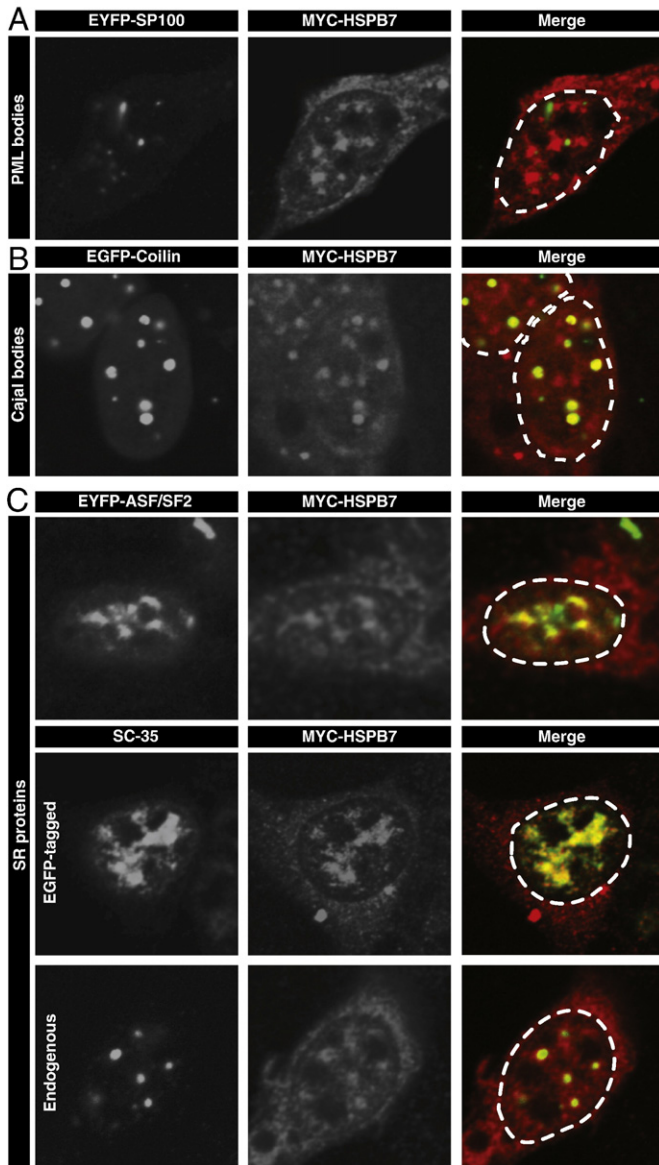


Fig. 5. HSPB7 associates with SR protein-containing nuclear domains. Myc-HSPB7 was analyzed for colocalization with either (A) SP100; a marker for PML bodies, (B) Coilin; a marker for Cajal bodies or (C) SR proteins ASF/SF2 and SC35; two markers of splicing speckles. Co-localization with SC35-EGFP was confirmed by staining for endogenous SC35.

complete co-localization with MYC-HSPB7 (Fig. 5C). Similarly, EGFP-tagged SC35 also completely co-localized to MYC-HSPB7 (Fig. 5C). Finally, staining of endogenous SC35 confirmed co-localization with MYC-HSPB7 (Fig. 5C).

So, in contrast to HSPB1 and HSPB5 that enter the nucleus and associate with speckles only after stress, HSPB7 localizes to these splicing factories also in non-stressed human HEK293 cells (Fig. 4B). To test whether this localization is not restricted to HEK293 cells only, we also expressed V5-HSPB7 in mouse HL-1 cardiac myocytes and human HeLa cells. Also in these two cell types HSPB7 localized to nuclear speckles (Fig. 6A, B), whereas V5-HSPB1 was largely cytosolic in both cell types under non-stressed conditions (Fig. 6C, D).

3.4. The N-terminus of HSPB7 is required and sufficient for localization to SC35 splicing speckles

As the conserved crystallin domain is the conserved motif for the HSPB members, it is likely that sub-cellular localization is regulated by targeting sequences present in the N-terminal or C-terminal domain.

Indeed, for *Drosophila* HSP27 and HSP22 a nuclear localization signal and a mitochondrial targeting sequence respectively have been identified in the N-terminal domains [54,58]. To analyze if the N-terminus of HSPB7 is required for speckle association, we deleted the first 71 amino acids of HSPB7 (the Δ N-HSPB7 mutant, Fig. 7A). Compared to the full length HSPB7 (Fig. 7B1, B1'), the Δ N-HSPB7 mutant showed less nuclear staining with a complete loss of speckle association (Fig. 7B2, B2'). To test whether the N-terminus of HSPB7 indeed drives speckle localization, we generated HSPB7 chimeras by switching the N-terminal domains between HSPB1 and HSPB7 (Fig. 7A). When expressed in cells, HSPB7 containing the N-terminus of HSPB1 (NB1- Δ NB7) was present in both the cytoplasm and nucleus but completely lacked any detectable association with sub-nuclear domains (Fig. 7B3, B3'). HSPB1 containing the N-terminus of HSPB7 (NB7- Δ NB1) on the other hand showed staining of sub-nuclear structures resembling wildtype HSPB7 (Fig. 7B4, B4'). Staining of endogenous SC35 indeed confirmed association of NB7- Δ NB1 with speckles (Fig. 7C). Thus it seems that speckle association of HSPB7 is indeed dependent on the N-terminal domain. To test whether the N-terminus of HSPB7 can also target proteins without a crystallin domain to SC35 speckles, we fused the N-terminus of HSPB7 to the red fluorescent protein mRFPPruby (NB7-mRFPPruby). The NB7-mRFPPruby chimeric protein indeed co-localized with EGFP tagged SC35 (Fig. 7D), proving that the N-terminus of HSPB7 contains a novel nuclear speckle localization signal which functions independently of HSPB domain architecture.

3.5. Speckle association of HSPB1 and HSPB7 is not linked to chaperone activity

In previous studies [9,10] it was found that after heat shock, not only chaperones like HSPB1 are reallocated to SC35 speckles, but also heat-denatured proteins. Although HSPB1 overexpression could enhance refolding of heat-denatured nuclear model substrates like firefly luciferase, indirect evidence, including the absence of HSP70 family members in these foci [63], suggested that the association of HSPB1 and denatured luciferase in SC35 speckles was not associated with refolding activity. To further look into the relevance of the

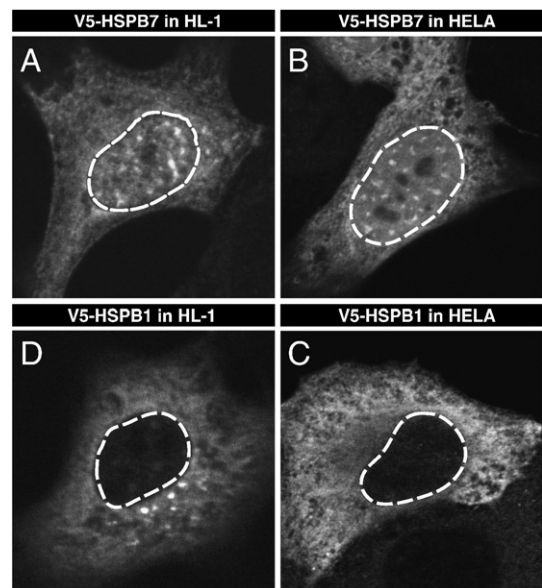


Fig. 6. HSPB7 association with nuclear domains is cell-type independent. V5-HSPB7 was transfected and visualized in either (A) mouse HL-1 or (B) human HeLa cells. (C, D) In both cell lines V5-HSPB1 was predominantly cytoplasmic. The nucleus was visualized by expression of the human Histone-2B fused to the monomeric red fluorescent protein ruby (H2B-mRFPPruby). This signal was traced in Adobe Photoshop and depicted as a dashed line.

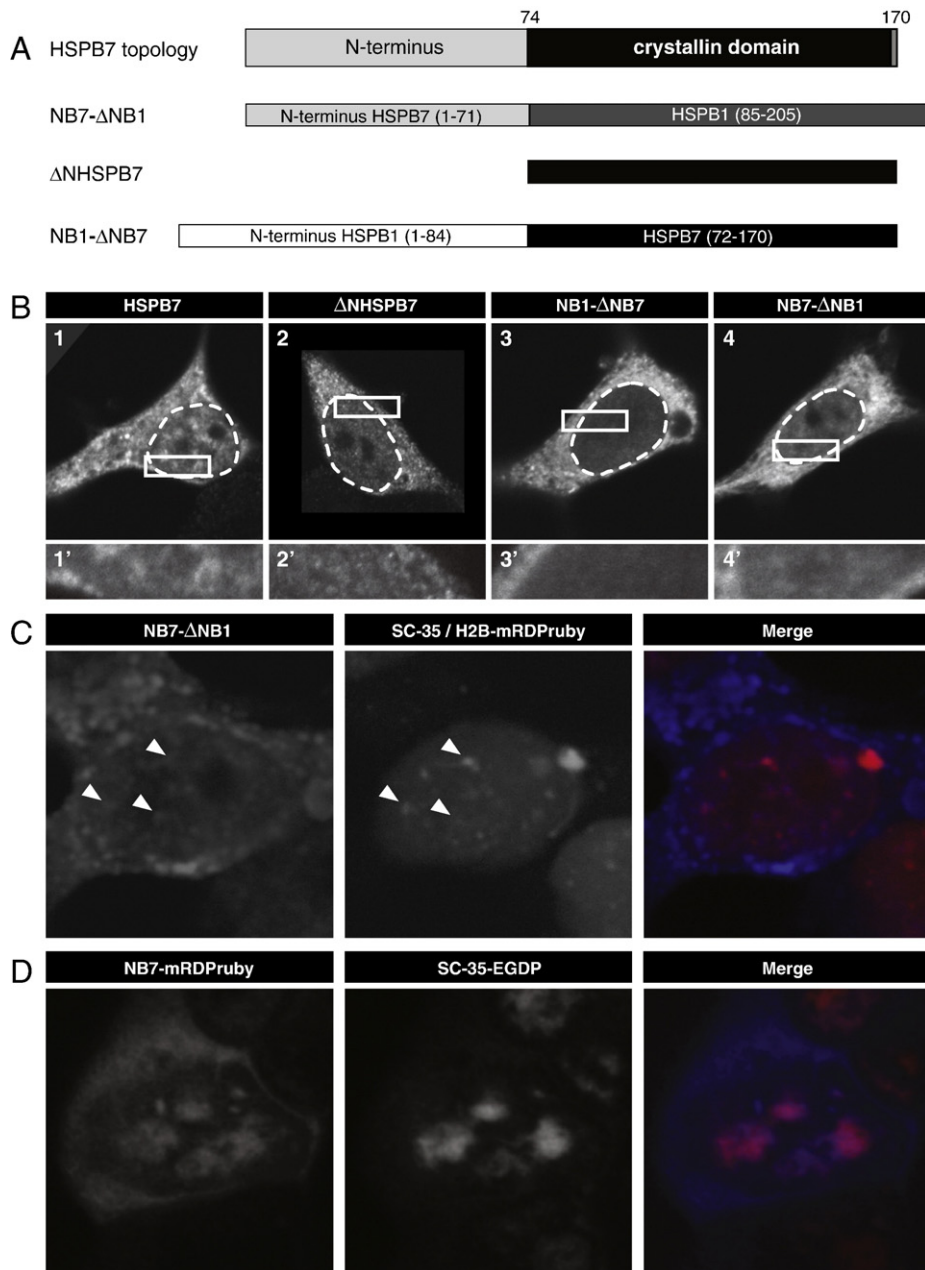


Fig. 7. The HSPB7 N-terminus is required and sufficient for targeting to SC35 speckles. (A) Schematic representation of the topology of HSPB7 and the constructed mutant and chimeric proteins. Localization pattern and close-up of HSPB7 (B 1/1'), Δ NHSPB7 (B 2/2'), NB1- Δ NHSPB7 (B 3/3') and NB7- Δ NHSPB1 (B 4/4'). (C) Staining of NB7- Δ NHSPB1 together with endogenous SC35 shows co-localization of both proteins. The nucleus was visualized using H2B-mRFP. (D) Expression of a chimeric protein containing the N-terminus of HSPB7 fused to mRFP localized to SC35-EGFP stained speckles.

presence of HSPB7, HSPB1 and unfolded luciferase in SC35 speckles for the storage and subsequent processing of unfolded nuclear proteins, we first tested whether the constitutively SC35-associated HSPB7 also could assist in the refolding of heat-denatured nuclear localized luciferase. Overexpression of either V5-tagged HSPB1, HSPB4 or HSPB5 indeed resulted in enhanced refolding (Fig. 8A) as described before for the untagged versions *in vitro* [39,45]. Refolding activity of HSPB4 towards nuclear luciferase was due to a translocation of HSPB4 towards the nucleus upon heat shock (Fig. S2a, S3). Under these conditions, NLS tagged luciferase was found exclusively in the nucleus (Fig. S3), suggesting that the cytosolic fraction of HSPB4 does not contribute to refolding of heat-denatured nuclear luciferase. Overexpression of the SC35-associated V5-HSPB7 did however not show any refolding promoting activity (Fig. 8A). The NB1- Δ NB7 mutant, harboring the crystallin domain of HSPB7 and devoid of nuclear

speckle association also did not show refolding promoting activity (Fig. 8B), in line with the complete lack of chaperone activity of the full length HSPB7. The NB7- Δ NB1 mutant, harboring the crystallin domain of HSPB1, but now associated with SC35 speckles via the N-terminus of HSPB7, lost refolding promoting activity (Fig. 8B); suggesting that localization in part results in loss of refolding capacity.

Finally, we asked whether speckle-associated HSPB proteins with no folding activity could affect the wildtype HSPB1 folding capacity via e.g. co-oligomerization or induced association with splicing speckles or by competitive substrate binding. Hereto, we co-expressed HSPB7 with HSPB1 and nuclear luciferase. This however did not affect the capacity of HSPB1 to assist in refolding heat-denatured luciferase (Fig. 8C). Next, we co-expressed the NB7-HSPB1 (NB7-B1) mutant, which is targeted to nuclear speckles, together with wildtype HSPB1. Again, HSPB1 refolding capacity was not negatively influenced by co-

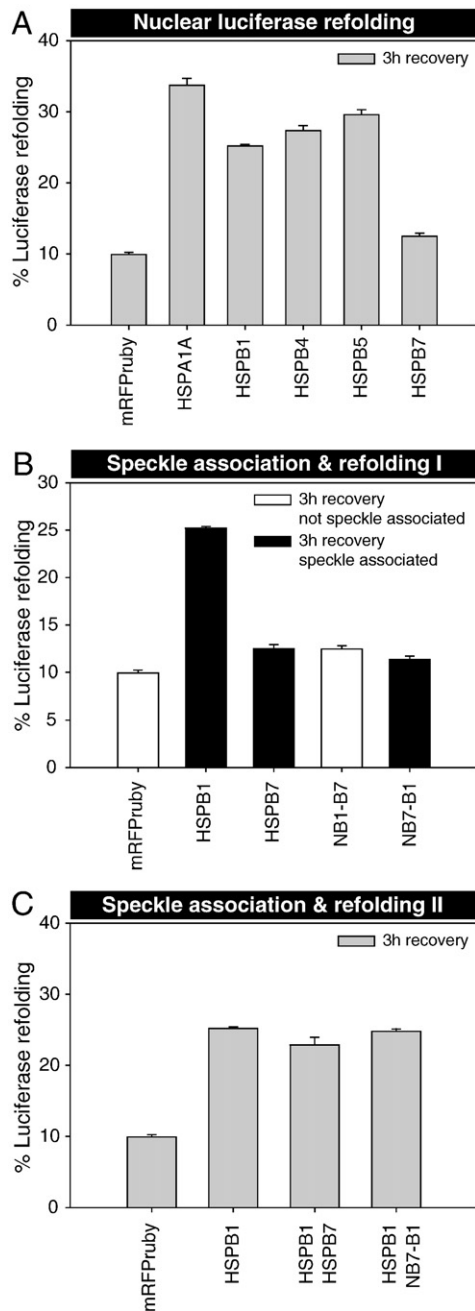


Fig. 8. HSPB7 and chaperone activity. HSPB proteins were co-expressed with nuclear firefly luciferase in HEK293 cells. 48 h after transfection, cells were heated for 30 min at 43 °C reducing luciferase activity to less than 5%. Next, cells were re-incubated for 3 h at 37 °C to allow for (chaperone-assisted) luciferase refolding. Luciferase activities are plotted relative to the activity in unheated cells (=100%). (A) Refolding of heat-denatured luciferase in cells overexpressing V5-HSPA1A (positive control), V5-HSPB1, V5-HSPB4, V5-HSPB5 and V5-HSPB7. (B) Comparison of chaperone activity in refolding heat-denatured luciferase of either SC35 speckle localized or non-SC35 speckle localized HSPB proteins. (C) Chaperone activity of HSPB1 without or with co-expression of resident speckle-associated HSPB proteins. Data are the mean \pm SD.

expression of a speckle-associated (mutant) HSPB protein (Fig. 8C). This highly suggests that NB7-B1, lacking folding activity, does not interfere with the folding-active wildtype HSPB1.

4. Discussion

We have presented an overview on several aspects and properties of the human HSPB family using a variety of specialized databases and focused subsequently on sub-cellular localization patterns. Apart from

their cytoskeletal localization, an issue not addressed in this manuscript, some HSPB members have been reported to reside in the nucleus. In fact, we now show that most HSPB members at least partially reside in the nucleus during non-stressed states some being associated with sub-nuclear structures called SC35- or splicing speckles (HSPB5 and HSPB7). Even more so, during and after heating all HSPB members show nuclear localization, often in foci, which for HSPB1, HSPB5 and HSPB7 were demonstrated to be SC35 speckles. Finally, we for the first time show that HSPB7 contains a speckle targeting sequences in its N-terminus that is capable of driving it and other proteins to splicing speckles under non-stressful conditions.

4.1. HSPB7 is a resident component of SC35-speckles containing a speckle targeting sequence in its N-terminal domain

SC35 speckles (also known as nuclear splicing speckles) are sub-nuclear structures involved in the storage and assembly of the splicing machinery and contain pre-mRNA splicing factors, transcription factors, snRNAs and ribosomal proteins [53]. Furthermore, modulation in splicing factor concentrations appears to link SC35 speckles to alternative splicing [1]. The SC35 speckle resident splicing factors primarily belong to the serine/arginine-rich (SR) protein family [15,38].

In general, little is known on how proteins are able to associate with SC35 speckles. One known SC35 speckle targeting signal consists of the SR motif itself, as was shown for the SC35 protein [11], although the presence of an SR motif not always drives proteins to SC35 speckles, meaning that other elements or the context around SR motifs may also play a role [72]. Until now, only one non-SR motif has been reported to function as a targeting signal for SC35 speckles. Cdk9, an elongation factor for RNA polymerase II-directed transcription and DYRK1A, a protein kinase [1,32], both contain a histidine-rich region which was shown to be sufficient to target GFP to SC35 speckles. HSPB1, HSPB5 and HSPB7 however lack such an SR motif and histidine-rich region, suggesting that other elements in the HSPB sequences are required for speckle association. The dependency on a specific sequence needed for SC35 speckle association was reported for the *Drosophila* HSP27 [54]. The authors showed that speckles association in HeLa cells was dependent on two leucine residues (L50, L52) located in the N-terminus. Although leucines are present in the N-termini of HSPB1, HSPB5 and HSPB7, their contribution to speckle association has yet to be established (Fig. S4).

In this study we showed that the N-terminus of HSPB7 functions as a unique and novel SC35 targeting sequence. Deletion of the N-terminus of HSPB7 or replacement by the N-terminus of HSPB1, abrogated HSPB7 speckle association. We furthermore showed that the N-terminus alone is sufficient to target either a crystallin domain or mRFP_{rubby} to SC35 speckles. HSPB7 contains a serine-rich region, which shows homology to the SR motif. Deletion of the serine stretch did however not result in dissociation of HSPB7 from SC35 speckles (data not shown). This suggests that the N-terminus of HSPB7 contains additional elements allowing for speckle association, which have yet to be determined.

4.2. Functional implications of HSPB speckle associations

HSPB1 is known to associate with SC35 speckles during heat stress. When luciferase is overexpressed under these conditions, it also associates with SC35 speckles [10]. In view of this, SC35 speckles might function as storage sites for HSPB-luciferase complexes, ready to be refolded upon stress relief. Could SC35 speckles indeed act as a depot for unfolded proteins, and does the association of HSPB proteins with SC35 speckles reflect a role in chaperoning of protein refolding? We showed that the SC35 speckle resident HSPB7 is not active in refolding of heat-denatured luciferase in contrast to HSPB1 and HSPB5. This suggests that at least for HSPB7, association to SC35 speckles is unrelated to protein refolding. By exchanging the N-

terminus of HSPB1 for the N-terminus of HSPB7, we were able to target the HSPB1 crystallin domain to SC35 speckles which resulted in loss of refolding activity. This suggests either that speckles association of HSPB1 is not related to refolding activity or that the HSPB1 N-terminus is required together with the HSPB1 crystallin domain to form a functional unit in assisting protein refolding activity. While co-expression of HSPB5 and luciferase clearly showed that this HSPB member is a chaperone capable of refolding heat-denatured luciferase, the direct association of HSPB5 with SC35 speckles has been suggested to relate to protein ubiquitination and degradation rather than protein refolding [18,20]. In conclusion, data on HSPB association with SC35 speckles hint to other functions than specific refolding of denatured proteins *in situ*.

As HSPB1 has been shown to enhance recovery of splicing after a heat shock, a role in splicing of HSPB7 at SC35 speckles should be addressed. A possible function of the SC35 speckle resident HSPB7 herein could be to fulfill a function in splicing under normal conditions while accumulation of HSPB1 at SC35 speckles during stress indicates a requirement to protect the splicing machinery and accelerate splicing recovery. This idea is under current investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbamcr.2009.05.005.

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