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Original research article

Hspb7 is a cardioprotective chaperone facilitating sarcomeric proteostasis

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ABSTRACT

Small heat shock proteins are chaperones with variable mechanisms of action. The function of cardiac family member Hspb7 is unknown, despite being identified through GWAS as a potential cardiomyopathy risk gene. We discovered that zebrafish *hspb7* mutants display mild focal cardiac fibrosis and sarcomeric abnormalities. Significant mortality was observed in adult *hspb7* mutants subjected to exercise stress, demonstrating a genetic and environmental interaction that determines disease outcome. We identified large sarcomeric proteins FilaminC and Titin as Hspb7 binding partners in cardiac cells. Damaged FilaminC undergoes autophagic processing to maintain sarcomeric homeostasis. Loss of Hspb7 in zebrafish or human cardiomyocytes stimulated autophagic pathways and expression of the sister gene encoding Hspb5. Inhibiting autophagy caused FilaminC aggregation in *HSPB7* mutant human cardiomyocytes and developmental cardiomyopathy in *hspb7* mutant zebrafish embryos. These studies highlight the importance of damage-processing networks in cardiomyocytes, and a previously unrecognized role in this context for Hspb7.

1. Introduction

Prevention, recognition and management of protein damage are essential cellular processes for physiological function at the level of the individual cell and the whole organ. This is clearly demonstrated by diseases that stem from pathologic aggregation of mutant or damaged proteins, or disturbance of these homeostatic regulatory pathways (Boncoraglio et al., 2012; Knowles et al., 2014; Wang and Robbins, 2006). Cytoprotective measures are particularly important in terminally differentiated, non-dividing cells that are more vulnerable to the toxic accumulation of misfolded or dysfunctional proteins (as they are unable to dilute them through cell division). Cell types that fall into this category include neurons and muscle cells, including cardiomyocytes. Myocytes must have particularly robust systems for turning over damaged proteins, since they contain large sarcomeric proteins undergoing constant strain and stress, for example in working skeletal muscle, or beating cardiac tissue. The importance of proteostasis is amplified in the non-dividing mammalian cardiomyocyte.

The small heat shock proteins (sHSPs) are a family of 15–30 kDa putative chaperones that are expressed throughout embryonic development and in adult organs. They have varied patterns of expression and heterogeneous mechanisms of action. While poorly understood compared to their higher molecular weight HSP counterparts, sHSPs have recently been shown to be important for maintaining cell fitness in a range of both physiological and pathophysiological conditions. These include a group of disorders associated with altered protein aggregation and cytoskeletal abnormalities including cataract formation (Litt et al., 1998), myopathies (Cappola et al., 2010; Stark et al., 2010; Vicart et al., 1998) and cancer (Zoubeidi and Gleave, 2012). The sHSPs share a conserved α-crystallin domain but exhibit large variation in expression patterns and mechanisms of action (Kappe et al., 2003; Ke et al., 2011; Morrow and Tanguay, 2012; Vos et al., 2010). They are putative chaperone proteins based on the α-crystallin domain, but the natural target substrates are generally unknown. Recently, three separate genome-wide association studies implicated polymorphisms in small heat shock protein, beta 7 (*HSPB7)* as potential contributors to idiopathic cardiomyopathies (Cappola et al., 2010; Stark et al., 2010; Villard et al., 2011).

HSPB7 is one of the least characterized sHSPs beyond recognition that it is highly expressed in the developing and adult heart (Elicker and Hutson, 2007; Krief et al., 1999). The structural and functional diversity seen within the sHSP family preclude prediction of HSPB7 function based on other sHSP family members. Recent studies characterizing the action of sHSPs reported that several family members prevent polyQ protein aggregation, including most potently HSPB7 (Vos et al., 2010). The efficacy of HSPB7 in preventing aggregation was striking, however, these were in vitro assays, and given that HSPB7 is expressed in striated muscle rather than in the brain, polyQ proteins are unlikely to be an endogenous target of HSPB7. It was also reported that HSPB7 co-localizes with

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tachypacing-induced F-actin stress fibers, and is protective against their formation in transformed cultured murine atrial cardiac (HL-1) cells (Ke et al., 2011). A recent publication demonstrated that the in vitro cytoprotective anti-polyQ action of HSPB7 occurs before the formation of aggregates, suggesting that HSPB7 either attenuates protein damage or facilitates early processing of damaged proteins (Eenjes et al., 2016).

However, the physiological target(s) and molecular mechanism(s) of action for HSPB7 in the heart remain obscure. We reported that *hspb7* lies downstream of the key cardiac transcription factor Gata4 in the developing zebrafish heart, and that *hspb7* depletion leads to defects in cardiomorphogenesis (Rosenfeld et al., 2013). Gata4 has been shown to be cardioprotective (Kitta et al., 2003) and it seemed feasible that Hspb7 could be a mechanistic component in this context. Therefore, we generated *hspb7* homozygous mutant zebrafish and characterized heart phenotypes, including an adult cardiac pathology that leads to a predisposition to exercise-induced death. We discovered evidence of upregulation and strict dependency on autophagy in mutant hearts and discovered FilaminC (FLNC) as a novel binding partner of HSPB7. We propose a model in which HSPB7 normally facilitates early post-damage processing of large sarcomeric cytoskeletal proteins, and loss of HSPB7 function leads to increased damaged protein load, protein aggregation, and risk for cardiomyopathy.

2. Materials and methods

2.1. Zebrafish husbandry

All zebrafish strains were maintained at 28.5 °C in our zebrafish facility, and all procedures carried out as approved by the WCMC IACUC. Embryos were staged as described (Westerfield, 1993). The *tg(myl7:gfp)* strain was originally obtained from H.J. Tsai (Taiwan).

2.2. Generation of hspb7 mutant zebrafish lines

Sequences of TALEN and CRISPRs used in this study are provided in Table 1. TALENs were designed using the Cornell TAL Effector Nucleotide Targeter 2.0 website (Doyle et al., 2012) and plasmids encoding specific TALENs were generated with the TALEN Golden Gate 2.0 kit (Addgene), as previously described (Cermak et al., 2011). The mRNA encoding TALENs was synthesized with the mMessage mMachine kit (Thermo Fisher) and 100–400 pg was injected into zebrafish embryos at the 1–2 cell stage. Guide RNAs (gRNAs) for CRISPR-Cas9 mediated mutagenesis were designed with the CHOPCHOP website (Montague et al., 2014) and ordered as double stranded oligo cassettes with a T7 promoter from IDT. The gRNA was generated with the MEGAshortscript kit (Thermo Fisher) and 250 pg was injected with 500 pg recombinant Cas9 protein (PNA BIO) into embryos at the 1–2 cell stage. Carriers of mutations were identified through PCR across the target locus followed by RFLP analysis or re-naturation of PCR products followed by digestion with T7 endonuclease I (NEB). PCR products were cloned into a TOPO vector (Invitrogen) and sequenced to identify mutant alleles.

2.3. Zebrafish stress test

Equal numbers of *hspb7* mutant zebrafish and wildtype sibling controls were placed in a swim tunnel (Loligo Systems, Denmark) and subjected to water flow of 50 cm^{-1} for 30 min, once a day. Zebrafish mortality was tracked by physical daily count, and fish locations tracked with an iPhone camera. Zebrafish location during swim challenge was tracked with MTrackJ for ImageJ (Meijering et al., 2012).

2.4. Anatomical analysis of adult zebrafish

Adult male zebrafish were submitted for histopathological processing and blinded analysis by the pathology core at Memorial Sloan Kettering Cancer Center. The fish were fixed in Bouin's solution for 24 h, cut parasagitally, processed via automated tissue processor, embedded in paraffin, and two 4 μm sections were taken 100 μm apart. Sections were then stained with hematoxylin and eosin and Masson's trichrome using standard methods. Adult zebrafish hearts were dissected as described (Arnaout et al., 2014) and fixed in 2.5% glutaraldehyde, 4% paraformaldehyde, (both from Electron Microscopy Biosciences), and 0.02% picric acid (Thermo). Samples were submitted to the Microscopy and Image Analysis Core at Weill Cornell Medicine. Following post-fixation processing as described (de Bruijn, 1973), samples were contrasted with lead citrate and viewed on a JEM 1400 electron microscope (JEOL, USA, Inc. Peabody, MA) operated at 100 kV. Digital images were captured on a Veleta 2 K × 2 K CCD camera (EM-SIS, Germany). Sectioned heart samples were imaged and analyzed cumulatively from 3 independent 18 month wildtype or *hspb7* mutant fish. The total area analyzed was 3405 μm^2 and 3738 μm^2 for the wildtype and the mutant hearts, respectively.

2.5. Measuring zebrafish heart size

Hearts were dissected from adult zebrafish as described (Ding et al., 2011). Following dissection, adult zebrafish hearts were placed on a microscope slide and imaged on a dissection microscope (Nikon) with top-lighting. Hearts were rotated to present maximal ventricular area and images were captured with Spot Imaging Software. Ventricular area was calculated with FIJI software (www.fiji.sc/). Prior to dissection, anesthetized zebrafish were partially dried with paper towels and weighed. Zebrafish mass was used as a normalizing factor for heart size index (VA/BW) since it is a greater variable than body length in adult fish.

2.6. Small molecule treatment of embryonic zebrafish

Wildtype, *hspb7* mutant embryos, or wildtype embryos injected with a previously validated (Rosenfeld et al., 2013) *hspb7* morpholino (CCTGTTCTGTCTGATGAAAAACATA, Gene Tools) were manually dechorionated with forceps at 24 hpf and exposed to increasing concentrations of bafilomycin A1, chloroquine diphosphate or 3-methyladenine (all from Sigma) in a solution of E3 medium (5.0 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl, 0.33 mM $MgSO_4$) with 1% DMSO (Sigma). 1 nl (0.9 mM) of morpholino was injected at the one cell stage.

Table 1
sgRNA sequences and TALEN targeted sequences in fish and human *HSPB7* genes.

Gene	**Species**	**gRNA Targeted Sequence (PAM)**	
hspb5b	Zebrafish	5′-ACGTGATCTCCTCATTGTAC(**TCC**)-3′	
HSPB7	Human	5′-(**CCC**)ACTCGGAGCCCCTGGCCTTC-3′	
Gene	**Species**	**Targeted Sequence**	**RVD sequences**
hspb7	Zebrafish	5′-CCTCCTCATCTTCATCCTCT-3′	HD HD NG HD HD NG HD NI NG HD NG NG HD NI NG HD HD NG HD NG
		5′-TACATGGAGAAGAGCCGAGG–3′	HD HD NG HD NN NN HD NG HD NG NG HD NG HD HD NI NG NN NG NI