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Original Article

The Prerequisite of Nuclear Localization for Heat Shock Protein 27 in Modulation of Starvation Adaptation and Longevity in *Drosophila*

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Heat shock protein 27 (HSP27) is a molecular chaperone adapting to stresses and sustaining physiological functions. Neuronal or ubiquitous overexpression of HSP27 which is located in nucleus can enhance stress resistance and extend lifespan in *Drosophila*. However, whether the nuclear localization of HSP27 is required for enhanced stress response and longevity remains unknown. Here we report that nuclear localization of HSP27 plays an important role in starvation response and longevity upon overexpression in *Drosophila*. Site-directed mutagenesis of three arginine residues responsible for HSP27 nuclear localization to glycines excludes mutant HSP27 in nucleus and instead accumulates in cytoplasm. While overexpressing wild-type HSP27 confers starvation resistance, overexpression of the mutant HSP27 fails to retain the enhanced starvation tolerance in the transgenic flies. Overexpressing the mutant HSP27 in neurons or ubiquitously loses the ability to extend lifespan in *Drosophila*. Together, the results shed a light on the importance of nuclear localization of HSP27 in modulating starvation response and longevity in *Drosophila*. The further identification of nuclear HSP27 interacting proteins accountable for starvation adaptation and longevity will be next to pursue.

Key Words: aging, HSP27, stress response

Introduction

Molecular chaperones adapt to environmental stresses to maintain protein homeostasis and uphold physiological functions (7). Heat shock proteins (HSPs) are molecular chaperones in response to environmental stresses to maintain protein folding or eliminate irreversibly damaged proteins by energy-dependent proteases (5, 21, 25). There are four small *hsp* genes, *hsp22*, *hsp23*, *hsp26*, *hsp27*, located at locus 67B in *Drosophila* genome (3). Although they are highly similar in sequence, they present distinct activities *in vitro* (16). *Drosophila* small HSPs have different intracellular localizations, respectively localized in cytoplasm (HSP23 and HSP26), mitochondria (HSP22) and nucleus (HSP27) (4, 12, 14, 17). The sequence for targeting HSP22 to mitochondria depends on signal peptide located in amino terminal, and the sequence for the nuclear localization of HSP27 relies on a arginine-rich nuclear localization signal (13).

Overexpression of *hsp22*, *hsp23*, *hsp26*, *hsp27* ubiquitously or tissue specifically have been shown to extend lifespan and enhance stress tolerance in *Drosophila* (2, 9, 18, 19, 23). Absence of small heat shock protein *hsp22* decrease lifespan in *Drosophila* (15). Moreover, the null mutant or knockdown of *hsp27* lead to decreased lifespan and starvation intolerance (2, 6). Though the intracellular localization of HSP27 is known in nucleus, yet the effect of intracellular localization of HSP27 on lifespan and stress response is not reported. In this study, we demonstrate for the first time that nuclear localization of HSP27 is prerequisite for HSP27-mediated starvation response and longevity in *Drosophila*.

Materials and Methods

Expression Constructs

The pMT-*hsp27*/*V5* expression construct was generated by cloning *hsp27* full-length cDNA with PCR amplification using the forward primer (hsp27-f):5'-CGCGAATTCTTGTCTAGACAGGGTTGT-3' and the reversed primer (hsp27-r):5'-CATCTCGAGCTTGCTAGTCTCCATTTTC-3' into the *EcoRI* and *XhoI* linkers of pMT/V5-HisA vector (Invitrogen) in frame with V5 tag. Site-specific mutations were generated using a two-step overlapping PCR approach (13). Two fragments, amino-terminal and carboxyl-terminal of *hsp27* with overlapping on 24 base-pairs over the residues to be mutated, were generated first. The amino-terminal fragment was generated with the forward hsp27-f primer and the reversed primer (Hsp27 R3G rev): 5'-CGAATAACCACCACCACCCAGTCC-3'. The carboxyl-

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terminal fragment was generated with the forward primer (Hsp27 R3G for): 5'-GGACTGGGTGGTG-GTGGTTATTCG-3' and the reversed hsp27-r primer. Each PCR-generated fragment was purified and used as a template in another PCR with hsp27-f and hsp27-r primers to generate the full-length cDNA of *hsp27* that incorporated three arginine→glycine substitutions in R54-56 residues. Then the mutant form of *hsp27* (named as $hsp27^{\Delta NLS}$) was also inserted into the *EcoRI* and *XhoI* sites of pMT/V5-HisA vector in frame with V5 tag. Both constructs were verified by DNA sequencing to ensure that the mutant $hsp27^{\Delta NLS}$ carries the correct mutations and no other mutation occurs in both constructs. The $hsp27^{\Delta NLS}$ was subcloned to *pINDY6* transgenic vector (23) and the resultant construct was used to generate the *UAS-*$hsp27^{\Delta NLS}$ transgenic flies.

S2 Cell Culture and Transfection

The Schneider 2 (S2) cell line, a gift from Dr. Jui-Chou Hsu in the Institute of Molecular Medicine, was grown in Schneider's *Drosophila* Medium (GIBCO) supplemented with 10% fetal bovine serum, 1 U/ml penicillin and 1 μg/ml streptomycin (GIBCO) in a 25°C incubator. For transfection, about 1.0×10^6 cells were seeded in 2 ml complete medium in a 6-well tissue culture plate (Corning) and allowed to attach for overnight. The following solutions were prepared before performing transfection. Solution A: 2 μg of DNA in 100 μl SFM (serum free medium). Solution B: 5 μl of METAFECTENE (Biontex) solution in 100 μl SFM. Next the two solutions were combined, mixed gently, and incubated for 20 min at room temperature. The cells were washed gently once with 2 ml SFM and the DNA-lipid complexes were added into the cell suspension for incubation about 3-4 h. Next the transfection mixture was removed and replaced with 2 ml new complete medium. After one-day incubation, the cells were added copper sulfate directly to the culture medium to a final concentration of 500 μM to induce the expression and incubated for another 24 h before the next procedure.

Immunocytochemistry

The copper sulfate induced transfected S2 cells were suspended to attach on poly-D-lysine coated cover slides for one hour, the attached cells were washed 3 times in ~~1XPBS~~ and fixed with ice-cold 4% paraformaldehye (PFA) for 30 min on ice. The fixed cells were washed 3 times with ice-cold ~~1XPBS~~ containing 0.3% Triton X-100, and incubated with anti-V5 antibody (Invitrogen, #96025) 1:500 diluted in PBS with 0.3% Triton X-100 and 3% BSA at 4°C for overnight. On the next day, the cells were washed 3 times with ~~1XPBS~~ containing 0.3% Triton X-100 and incubated with anti-mouse IgG cy5 conjugate (1:500 dilution) at 4°C for 2 h. Finally, the cells were washed 4 times with ~~1XPBS~~ with DAPI added to the last wash for 15 min, and mounted in 75% glycerol and sealed with nail polish. The image was captured with Carl Zeiss LSM 5 PASCAL confocal microscope.

Western Blot

The copper sulfate induced transfected S2 cells were collected and homogenized with lysis buffer (20 mM HEPES, 75 mM NaCl, 2.5 mM $MgCl_2$, 0.1 mM EDTA, 0.5% Trition X-100, 0.1 mM Na_3VO_4, 50 mM NaF), incubated on ice for 30 min and centrifuged with 12,000 rpm for 25 min at 4°C as previously described (10). The supernatant was collected and the protein concentration was determined. Equal amounts of the protein lysates were resolved on a 12% SDS-PAGE and transferred to a nitrocellulose membrane. Followed blocking with 5% non-fat skim milk in TBS-T (Tris-Buffered Saline containing 0.1% Tween 20), the membrane was incubated with the primary antibody (anti-V5, Invitrogen) at 4°C for overnight. Next the membrane was washed TBS-T three times and probed with HRP-conjugated secondary antibody (goat anti-mouse IgG, Zymed) at 4°C for 3 h, washed again with TBS-T three times, and incubated with ECL reagent following exposure to the X-ray film (Kodax) to grasp the image.

Fly Maintenance, Lifespan and Starvation Assays

All flies were maintained in the standard fly food (agar 0.86%, cornmeal 6.75%, dextrose 5.5%, yeast 2.83%, sucrose 2.79%) at 25°C, 65% humidity, under a 12 h/12 h light/dark cycle incubator as previously described (11, 22). For lifespan assay, thirty male flies for each were collected in each vial with fly food and maintained at 25°C. The flies were transferred to a new vial every 3 or 4 days and the number of dead flies were counted until all dead. The survival curve was plotted and the statistical significance was calculated by log-rank (Mantel-Cox) test. For starvation assay, twenty male flies about 3-day-old were collected in each vial and recovered overnight. The flies were transferred to the new vials with 1% agar gel. The numbers of dead flies were recorded every 4 h and the vials were replaced every day until all of them died as described previously (2). The statistical significance was determined by student's *t*-test.

Results

The Arginine Residues in Amino Acid 54 to 56 of HSP27 Is Required for Its Nuclear Localization

The amino acid sequence for HSP27 nuclear targeting

Fig. 1---Color

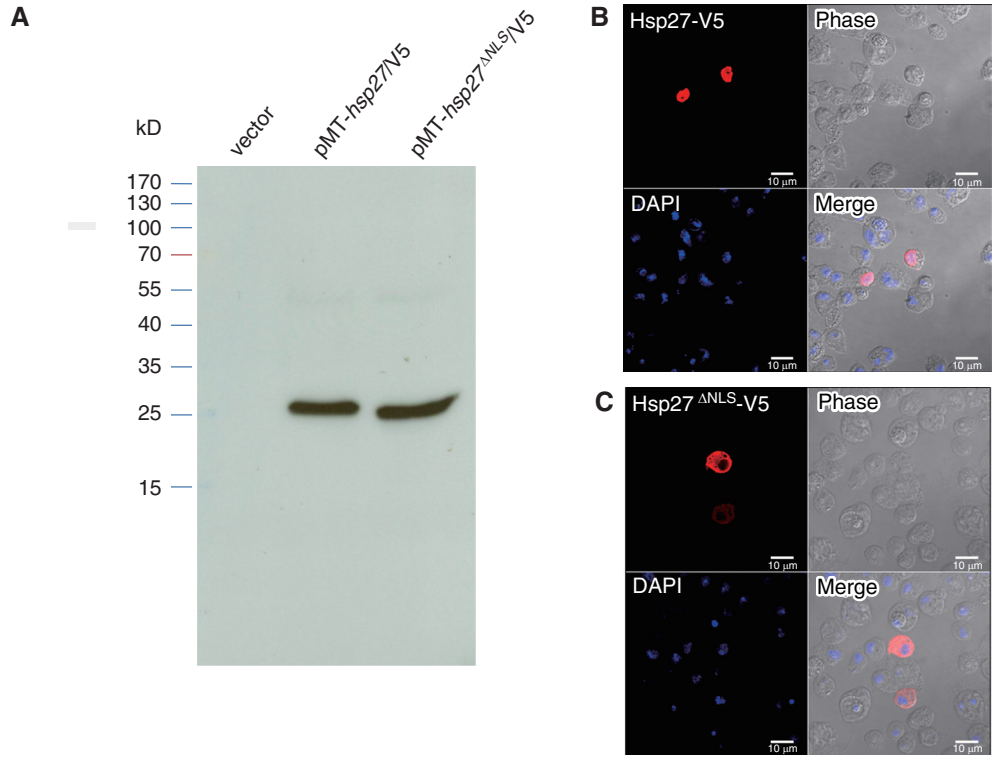

Fig. 1. **The intracellular localizations of wild-type and mutant HSP27 in *Drosophila* S2 cells.** The S2 cells were transfected with the constructs expressing V5-tagged HSP27 or HSP27$^{\Delta NLS}$ and applied in the western blot and immunocytochemistry. (A) Wild-type HSP27 and mutant HSP27$^{\Delta NLS}$ with V5 tag were expressed in S2 cells and detected by anti-V5 antibodies in the western blot. Mutant HSP27$^{\Delta NLS}$ is 0.3 kD lighter than wild-type HSP27 as the molecular weight of glycine is smaller than that of arginine. (B) Wild-type HSP27 is located in the nucleus of S2 cells. The V5-tagged HSP27 was detected by anti-V5 (red, cy5, left upper panel) and colocalized with nuclei by DAPI staining (blue, left lower panel) as shown in the merge photo (right lower panel). (C) Mutant HSP27$^{\Delta NLS}$ is excluded from nuclei and expressed in the cytoplasm in S2 cells. Expression of mutant V5-tagged HSP27$^{\Delta NLS}$ (red, cy5, left upper panel) did not overlap with DAPI staining (blue, left lower panel) as shown in the merge photo (right lower panel).

was identified in the arginine rich residues between amino acid 54-57 of HSP27 (13). Therefore, we mutated the three arginine residues 54-56 to become glycine in HSP27 in frame with V5 tag in the pMT/V5-HisA expression vector (pMT-hsp27$^{\Delta NLS}$/V5) and examined whether the nuclear localization is abolished in mutated HSP27$^{\Delta NLS}$/V5 compared with the wild-type HSP27 with V5 tag construct (pMT-hsp27/V5). First, we checked whether both constructs express HSP27/V5 and HSP27$^{\Delta NLS}$/V5 proteins in *Drosophila* S2 cells by western blot. By using anti-V5 antibodies, the results showed both constructs expressed V5 tagged HSP27 and HSP27$^{\Delta NLS}$ (Fig. 1A). As expected, the size of mutant HSP27$^{\Delta NLS}$/V5 is a little smaller than wild-type HSP27$^{\Delta NLS}$/V5 due to the lighter molecule weight of glycine than arginine. Next, we examined the cellular localization of both wild-type and mutant HSP27 proteins in *Drosophila* S2 cells by immunocytochemistry. The data indicated that wild-type HSP27 is located in nucleus (Fig. 1B) and mutant HSP27$^{\Delta NLS}$ is excluded from nucleus and expressed in cytoplasm (Fig. 1C). The results demonstrated that the substitution of the three arginines with glycines is effective to abrogate the nuclear localization of HSP27.

Nuclear Localization of HSP27 is Required for Enhanced Resistance to Starvation Stress

To examine the physiological function of the mutated HSP27$^{\Delta NLS}$ *in vivo*, we subcloned the mutant form of *hsp27*$^{\Delta NLS}$ into *pINDY6* transgenic vector to generate UAS-*hsp27*$^{\Delta NLS}$ transgenic flies. Our previous studies reported that overexpression of *hsp27* exhibits enhanced resistance to starvation stress in *Drosophila* (2, 23). We examined the effect of overexpression of the mutant HSP27$^{\Delta NLS}$ in comparison with the wild-type HSP27 on the starvation resistance. Overexpression of *hsp27* displayed an increase of 17.8% ($P < 0.001$) and 19.3% ($P < 0.001$) in mean survival time compared to the controls either UAS or Gal4 alone, respectively (Fig. 2A, Table 1). However, overexpression of the mutant form of *hsp27*$^{\Delta NLS}$ did not show the enhanced starvation

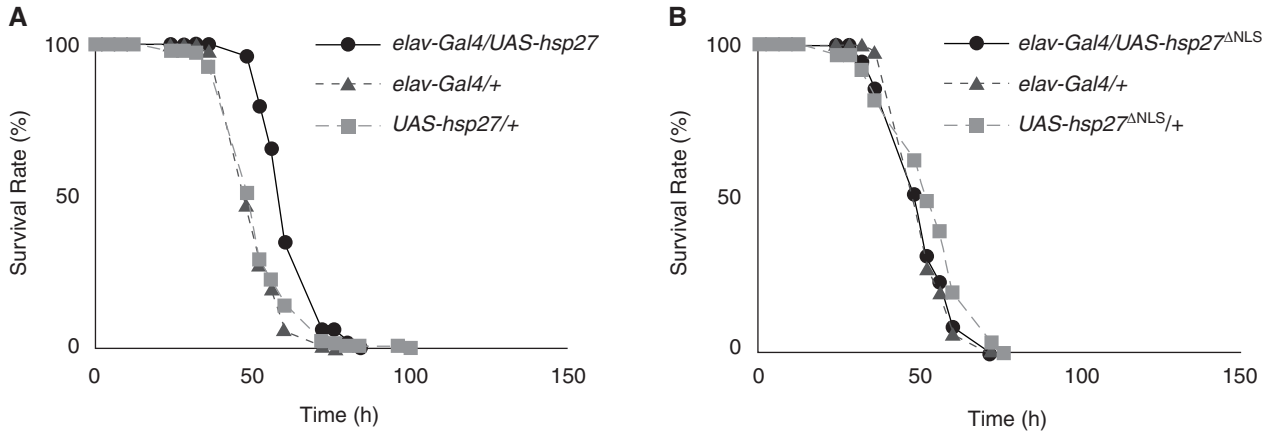

Fig. 2. **Nuclear form of HSP27 upon overexpression is required for enhanced resistance to starvation stress.** (A) Overexpression of *hsp27* by *elav*-Gal4 (triangle) displays an increase of 18% and 19% in mean survival time compared to UAS-*hsp27* (square) and *elav*-Gal4 (circle) alone respectively under starvation stress. (B) Overexpression of *hsp27*$^{\Delta NLS}$ by *elav*-Gal4 (circle) loses the enhancement and shows no differences in the survival time compared to both controls (UAS-*hsp27*$^{\Delta NLS}$, square; *elav*-Gal4, triangle) under starvation stress.

Table 1. The summary of starvation stress and lifespan assays by overexpression of HSP27$^{\Delta NLS}$ in *Drosophila*

The mean survival time in starvation stress test

Strain	Sample size	Mean ± SEM (h)	Difference, % compare to (Gal4/+)	Difference, % compare to (UAS/+)
elav-Gal4/UAS-*hsp27*	49	62.4 ± 1.6	19.3***	17.8***
UAS-*hsp27*/+	133	52.9 ± 2.7		
elav-Gal4/+	127	52.3 ± 1.9		
elav-Gal4/UAS-*hsp27*$^{\Delta NLS}$	35	51.3 ± 1.2	-1.9	-4.6
UAS-*hsp27*$^{\Delta NLS}$/+	154	53.8 ± 3.9		
elav-Gal4/+	127	52.3 ± 1.9		

P-value were calculated by Student's *t*-test: ***$P < 0.001$

The mean lifespan of the flies over-expressing hsp27$^{\Delta NLS}$

Strain	Sample size	Mean ± SEM (days)	Difference, % compare to (Gal4/+)	Difference, % compare to (UAS/+)
elav-Gal4/*hsp27*$^{\Delta NLS}$	73	40.6 ± 5.6	-9.6	-7.4
UAS-hsp27$^{\Delta NLS}$/+	113	43.8 ± 1.2		
elav-Gal4/+	230	44.9 ± 4.1		
hs-Gal4/*hsp27*$^{\Delta NLS}$	212	42.2 ± 3.7	-13.7***	-3.8
UAS-hsp27$^{\Delta NLS}$/+	113	43.8 ± 1.2		
hs-Gal4/+	228	48.9 ± 2.9		

P-value were calculated by log rank: ***$P < 0.001$

resistance ability (Fig. 2B, Table 1). It suggests that the nuclear localization of HSP27 is required for maintaining starvation tolerance in *Drosophila*.

Overexpression of the Mutant Form of HSP27ΔNLS Does Not Exhibit Extended Lifespan in Drosophila

Longevity is often associated with increased resistance to stress. Since loss of HSP27 nuclear localization in HSP27$^{\Delta NLS}$ upon overexpression lost the ability to cope with starvation stress, we next examined the effect of the mutant HSP27$^{\Delta NLS}$ overexpression on *Drosophila* lifespan. In our previous studies, overexpression of wild-type HSP27 in neurons or ubiquitously exhibits lifespan extension in *Drosophila* (2, 9, 23). To examine the effect of the mutant form HSP27$^{\Delta NLS}$ on lifespan, we expressed UAS-*hsp27*$^{\Delta NLS}$ in either neuronal tissue or ubiquitously driven by *elav*-Gal4 or

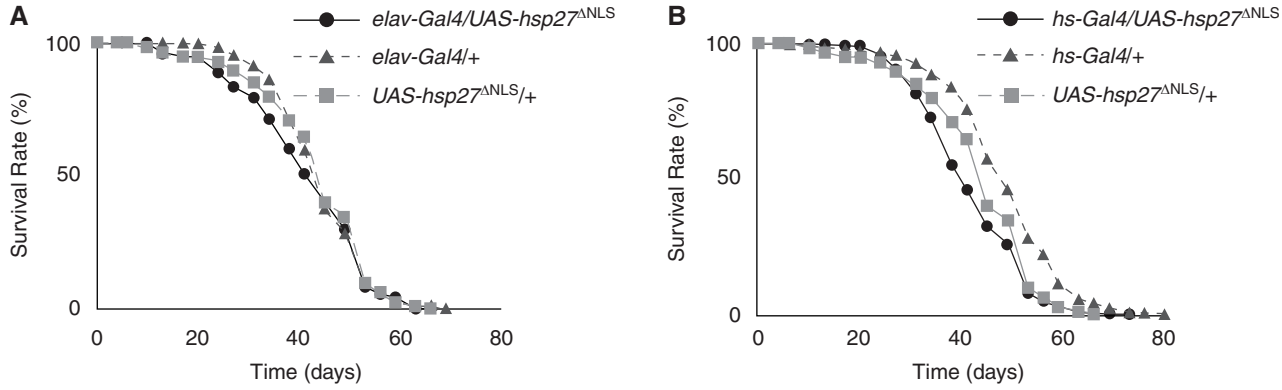

Fig. 3. **Overexpression of *hsp27*$^{\Delta NLS}$ by *elav*-Gal4 or *hs*-Gal4 does not show extended lifespan in *Drosophila.*** Overexpression of *hsp27*$^{\Delta NLS}$ by either (A) *elav*-Gal4 or (B) *hs*-Gal4 fails to extend lifespan in *Drosophila*. (circle, *hsp27*$^{\Delta NLS}$ overexpressing flies; square, UAS alone control; triangle, GAL4 alone control).

hs-Gal4 and measured their lifespan along with their controls. Consistent with the starvation results, no lifespan extension was detected by either the neuronal or ubiquitous expression of HSP27$^{\Delta NLS}$ (Fig. 3A, B). Moreover, ubiquitous expression of HSP27$^{\Delta NLS}$ by *hs*-Gal4 exhibited minor decreased lifespan when compared to Gal4 alone, but still no significant changes when compared to UAS alone (Fig. 3B, Table). Together, it suggests that the nuclear localization of HSP27 is required for the lifespan extension in *Drosophila* upon HSP27 overexpression.

In summary, ~~we~~ results demonstrate for the first time that nuclear localization is a prerequisite for HSP27 ~~overexpression~~ modulated starvation adaptation and longevity in *Drosophila*.

Discussion

There are 12 potential sHps containing alpha-crystalline in *Drosophila* genome (14). Four of them, *hsp22*, *hsp23*, *hsp26*, and *hsp27*, have been extensively studied and shown to extend lifespan in *Drosophila* upon overexpression. They show distinct intracellular localizations, with HSP22 in mitochondria matrix, HSP23 and HSP26 in cytoplasm, and HSP27 in nucleus. Whether the precise intracellular expression is required for HSP-mediated lifespan extension has never been reported. Our study demonstrates for the first time that the nuclear localization of HSP27 is required for HSP27-mediated starvation resistance and longevity in *Drosophila*.

Overexpression of HSP27 shows enhanced resistance to starvation and this ability relies on the nuclear localization of HSP27 in *Drosophila*. This suggests that nuclear HSP27 may interact with certain nuclear factors to promote starvation tolerance and even in longevity. HSP27 is in nucleus and associated to nuclear speckles where the subnuclear structures are located in the interchromatin regions and enriched in pre-messenger RNA splicing factors in *Drosophila* (8, 13). Phosphorylated HSP27 is recruited to nuclear speckles without stress in mammalian cells (1), suggesting that HSP27 has other function in addition to chaperone activity. According to the subnuclear localization, HSP27 may be involved in post-transcriptional modification. Alternatively, HSP27 may serve as an coactivator by interacting with other nuclear factors to modulate gene expression responsible for lifespan regulation. Several nuclear factors like FOXO and Nrf2 have been shown to regulate lifespan and stress response by modulating different panels of gene expression (20, 24). Our preliminary results have identified three potential nuclear factors, Ada2b, Slbo, and Ref(2)p, which interact with HSP27 in nucleus. Upon RNAi knockdown of *Ada2b*, *Slbo*, or *Ref(2)p*, the starvation resistance by HSP27 overexpression was abolished in *Drosophila* (unpublished data), suggesting that HSP27 may collaborate with those nuclear factors to modulate gene expression responsible for starvation response and maybe even in lifespan regulation. However, the details how and why overexpression of nuclear HSP27 may collaborate with those nuclear factors to promote starvation resistance and lifespan extension await for further experiments to verify. In summary, our studies shed a light on the importance of nuclear localization of HSP27 in modulating starvation response and longevity in *Drosophila*.

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