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Original article

Changes in *Drosophila* mitochondrial proteins following chaperone-mediated lifespan extension confirm a role of Hsp22 in mitochondrial UPR and reveal a mitochondrial localization for cathepsin D

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ABSTRACT

Hsp22 is a small mitochondrial heat shock protein (sHSP) preferentially up-regulated during aging in *Drosophila melanogaster*. Its developmental expression is strictly regulated and it is rapidly induced in conditions of stress. Hsp22 is one of the few sHSP to be localized inside mitochondria, and is the first sHSP to be involved in the mitochondrial unfolding protein response (UPR^{MT}) together with Hsp60, mitochondrial Hsp70 and TRAP1. The UPR^{MT} is a pro-longevity mechanism, and interestingly Hsp22 over-expression by-itself increases lifespan and resistance to stress. To unveil the effect of Hsp22 on the mitochondrial proteome, comparative IEF/SDS polyacrylamide 2D gels were done on mitochondria from Hsp22+ flies and controls. Among the proteins influenced by Hsp22 expression were proteins from the electron transport chain (ETC), the TCA cycle and mitochondrial Hsp70. Hsp22 co-migrates with ETC components and its over-expression is associated with an increase in mitochondrial protease activity. Interestingly, the only protease that showed significant changes upon Hsp22 over-expression in the comparative IEF/SDS-PAGE analysis was cathepsin D, which is localized in mitochondria in addition to lysosome in *D. melanogaster* as evidenced by cellular fractionation. Together the results are consistent with a role of Hsp22 in the UPR^{MT} and in mitochondrial proteostasis.

1. Introduction

Aging is a biological process characterized by a progressive loss of cellular integrity. While multiple roads lead to aging in different organisms, there are some common denominators such as genomic instability, loss of proteostasis, deregulated nutrient sensing, altered intercellular communication and mitochondrial dysfunction (Correia-Melo and Passos, 2015; Lane et al., 2015; Lopez-Otin et al., 2013; Rattan, 2010). Mitochondria are dynamic organelles at the cross-road of many metabolic pathways that are also involved in calcium homeostasis and apoptosis among other processes. As the main provider of ATP, their function is tightly regulated through constant communication with the nucleus allowing them to adapt to cellular needs (Haynes and Ron, 2010; Runkel et al., 2014; Schieke and Finkel, 2006). The maintenance of mitochondrial functions is therefore important for cellular homeostasis and, accordingly, the accumulation of dysfunctional mitochondria has been reported in numerous diseases as well as in aging (Correia-Melo and Passos, 2015; Lane et al., 2015; Lopez-Otin et al., 2013).

Among the mitochondria-to-nucleus signaling pathways is the mitochondrial unfolding protein response (UPR^{MT}),[4] which is

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[4] sHSPs: small heat shock proteins, UPR^{MT}: mitochondrial unfolding protein response, ETC: electron transport chain, mtHsp70: mitochondrial Hsp70, TRAP1: tumor necrosis factor receptor associated protein 1, TCA: tricarboxylic cycle,

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involved in maintaining mitochondrial proteostasis (Haynes et al., 2007; Haynes and Ron, 2010; Jovaisaite et al., 2014). This signaling pathway takes place when there is an accumulation of misfolded proteins in mitochondria and results in the up-regulation of mitochondrial chaperones and proteases. The main mitochondrial chaperones induced by UPR^{MT} are Hsp60 and the mitochondrial Hsp70 (mtHsp70). In *Drosophila melanogaster*, two other chaperones are also involved in UPR^{MT}, namely tumor necrosis factor receptor associated protein 1 (TRAP1) and Hsp22 (Baqri et al., 2014; Morrow et al., 2016; Shen and Tower, 2013; Tower, 2015; Tower et al., 2014).

D. melanogaster Hsp22 is one of the members of the small heat shock protein (sHSP) family and is constitutively localized inside mitochondria (Haslbeck and Vierling, 2015; Morrow et al., 2000; Morrow and Tanguay, 2015b). Unlike the other main sHSPs of Drosophila, its expression is tightly regulated and is observed during the metamorphosis of larvae to pupae and during adulthood (King and Tower, 1999; Michaud et al., 2002; Morrow and Tanguay, 2015b). Hsp22 acts as a molecular chaperone *in vitro* and its overexpression increases lifespan and resistance to stress, while its down-regulation is detrimental and sensitize flies to their environment (Hirano et al., 2012; Morrow et al., 2004a, 2004b; Moskalev et al., 2009). As a member of the fly UPR^{MT}, Hsp22 is up-regulated upon mitochondrial stress and recent data suggest that it could be involved in maintaining the UPR^{MT} signal (Morrow et al., 2016; Morrow and Tanguay, 2015a; Shen and Tower, 2013; Tower et al., 2014).

Ubiquitous over-expression of Hsp22 has been shown to influence the transcriptome of flies, affecting the levels of transcripts of proteins involved in electron transport and protein translation among others (Kim et al., 2010). In the present study we sought to investigate the effect of Hsp22 at the level of mitochondrial proteins. Hsp22 overexpression has a significant impact on the mitochondrial proteome of flies that can be observed as soon as day one in adults. The proteins up- and down-regulated in mitochondria from Hsp22 overexpressing flies includes proteins of the electron transport chain (ETC), the tricarboxylic cycle (TCA) as well as mitochondrial Hsp70 (mtHsp70), which is consistent with a role of Hsp22 in the UPR^{MT} (Fernandez-Ayala et al., 2010; Morrow et al., 2016; Morrow and Tanguay, 2015a; Owusu-Ansah et al., 2008; Shen and Tower, 2013; Tower et al., 2014). In line with a role of Hsp22 in this mitochondrial stress response, mitochondria from flies overexpressing Hsp22 have higher protease activity suggesting a better clearance of damaged proteins. Interestingly, Hsp22 expression is associated with high levels of cathepsin D, which is demonstrated for the first time to be constitutively localized in mitochondria of *D. melanogaster* in addition to its well-known lysosomal localization.

2. Experimental

2.1. Fly strains and maintenance

The *actin*-Gal4 strain (Flybase ID: FBti0012293) carries a P-element insertion containing the *actin5c* promoter in front of the *gal4* coding sequence on the second chromosome, which results in ubiquitous expression of the Gal4 protein. The EP(3)3247 strain (Flybase ID: FBti0011419, (Rorth, 1996)) contains an UAS-including P-element inserted 643 bp upstream of the *hsp22* translation initiation codon (Flybase ID: FBal0138481) on the third chromosome. Male siblings obtained from the crossing between *actin*-Gal4/*cyo* males and EP(3)3247/TM3,*sb* females were used. Activation of the UAS by GAL4 results in Hsp22 over-expression in flies carrying both P-elements (Morrow et al., 2004b). When Hsp22 over-expression was not needed, W^{1118} flies were used.

Flies were maintained at 25 °C on standard cornmeal-agar medium (0.5% agar, 2.7% yeast bakers dried active, 1.1% sugar, 5.3% cornmeal, 0.4% (v/v) propionic acid and 1.8% (v/v) tegosept) and transferred to fresh food every 3–4 days. For mitochondrial isolation, flies were used immediately without freezing.

For the comparative 2D IEF/SDS-PAGE experiment, 2000–4000 male flies *actin*-Gal4;EP(3)3247 (Hsp22+ flies) and *actin*-Gal4;TM3,*sb* (control flies) were collected separately within 48 h of hatching and transferred into tubes (20 flies per tube). Approximately 2000 flies were collected at 1, 25 and 55 days as shown in Fig. 1.

2.2. Mitochondrial isolation, NP-40 fractionation and proteinase K digestion

Crude mitochondria (CM) were isolated from whole flies using a slightly modified protocol (Melov et al., 1999). The procedure was optimized for 200 flies. Briefly, flies were placed on ice for 5 min, and were then homogenized with 7 ml of H-buffer (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, 5 mM HEPES, pH 7.2) in 15 ml conical tissue grinder (Tissue Grinder System, VWR international, USA). The homogenate was centrifuged twice at 1000 g during 5 min at 4 °C. Floating debris such as broken wings were removed from the resulting supernatant by filtration through 100 μm nylon sieves (Cell Strainer REF 352360, BD Falcon, USA), and the CM pellet was obtained from a 8800*g* centrifugation at 4 °C for 10 min and kept at −80 °C until use.

For NP40 fractionation, the fresh CM was resuspended in SEM (250 mM sucrose, 1 mM EDTA, 10 mM MOPS pH 7.2) and fractionated using 0.25, 0.5% or 1% NP-40 as described previously (Morrow et al., 2000).

For proteinase K experiments, the fresh CM pellet was separated in 4 different tubes and resuspended in SEM buffer with or without 5 μg proteinase K/ml and/or 0.1% SDS (Morrow et al., 2000). Following 1 h incubation on ice, proteinase K was inhibited with PMSF and the suspensions were centrifuged at 13,000 rpm. Both the pellets and the supernatants were loaded on a 12% SDS-PAGE. The experiment was also performed on the 8800*g* supernatant from the CM preparation to analyze the sensitivity of lysosomal cathepsin D to proteinase K. In this case, no centrifugation was required and suspensions were loaded on a 12% SDS-PAGE.

To obtain pure mitochondria (PM), the fresh CM were resuspended in ice-cold MRB (250 mM mannitol, 5 mM HEPES pH 7.4, 0.5 mM EGTA), layered on a 30% Percoll gradient (225 mM mannitol, 25 mM HEPES pH 7.4, 1 mM EGTA, 30% Percoll) and centrifuged at 95,000 *g* 30 min at 4 °C as described (Wieckowski et al., 2009). Two bands were visible after centrifugation. The light band, consisting of mitochondria-associated membranes (MAM), was diluted with MRB and centrifuged at 8800 *g* to remove mitochondrial contaminants. The supernatant was then pelleted at 100,000*g* to yield the MAM fraction (Wieckowski et al., 2009). The dense band, consisting of purified mitochondria (PM), was diluted in MRB, pelleted at 8800*g* and washed twice with MRB.

2.3. Western blots

Proteins were separated on 12% SDS-PAGE as described (Marin and Tanguay, 1996) and transferred on nitrocellulose membranes.

Hsp22+: flies over-expressing Hsp22, CM: crude mitochondria, PM: pure mitochondria, MAM: mitochondria-associated membranes, MW: molecular weight, CM-Pool: common pool containing equal amount of all six samples (C1d, C25d, C55d, H1d, H25d, H55d), ROS: reactive oxygen species, MALM: mieap-induced accumulation of lysosome-like organelles within mitochondria, C1d/C100%: 1 day-old/100% survival control flies, C25d/C90%: 25 days-old/90% survival control flies, C55d/C50%: 55 days-old/50% survival control flies, H1d/H100%: 1 day-old/100% survival Hsp22+ flies, H25d/H98%: 25 days-old/98% survival Hsp22+ flies, H55d/H90%: 55 days-old/90% survival Hsp22+ flies.