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TRANSCRIPTOMIC AND PROTEOMIC STUDIES ON LONGEVITY INDUCED BY OVER-EXPRESSION OF HSP22 IN DROSOPHILA MELANOGASTER

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Résumé

Le vieillissement est un processus complexe accompagné par une capacité diminuée des cellules à tolérer et répondre aux formes différentes de stress causant des dommages comme l'agrégation de protéine dans les différentes composantes de la cellule. Les chaperons sont des joueurs probablement importants dans le processus de vieillissement en prévenant la dénaturation et l'agrégation des protéines. Chez *Drosophila melanogaster*, une petite protéine de choc thermique, Hsp22, localisée dans la matrice mitochondriale montre une expression elevée pendant le vieillissement. Sa surexpression chez la mouche augmente la durée moyenne de vie ainsi que la résistance au stress. Bien que Hsp22 montre une activité de chaperon dans des essais *in vitro*, les mécanismes par lesquels Hsp22 permet d'accroitre la durée de vie *in vivo* sont toujours inconnus.

Une analyse transcriptionelle de tout le génome par microarrays et une analyse comparative du protéome mitochondrial par MALDI-TOF a été entreprise pour dévoiler les différences d'expression entre les mouches surexprimant Hsp22 et les contrôles appropriés. La surexpression générale de Hsp22 en utilisant le système GAL4/UAS dans *Drosophila* résulte en une augmentation de ~ 30% dans la durée de vie moyenne. L'analyse du transcriptome suggère que Hsp22 joue un rôle dans la détermination de durée de vie en changeant le processus général de vieillissement normal. Effectivement, les mouches surexprimant Hsp22 affichent une surexpression de gènes dont l'expression baisse normalement durant le vieillissement. Les gènes sont impliqués dans la production d'énergie, la biosynthèse des protéines, le taux de renouvellement des protéines et le métabolisme lipidique. L'analyse du protéome mitochondrial soutient aussi un rôle de Hsp22 sur la détermination de la durée de vie en maintenant la fonction mitochondriale et en favorisant la protéolyse. Les présentes données suggèrent l'importance de la maintenance de l'homéostasie protéique durant le vieillissement et discutent des mécanismes potentiels d'extension de la longévitié chez les mouches surexprimant Hsp22.

Abstract

Aging is a complex process accompanied by a decreased capacity of cells to tolerate and respond to various forms of stresses leading to damages such as protein aggregation in various components of the cell. Chaperones are thus likely important players in the aging process by preventing protein denaturation and aggregation. In *Drosophila melanogaster*, a small heat shock protein Hsp22 localized in the mitochondrial matrix is preferentially up-regulated during aging. Its over-expression results in an extension of lifespan and an increased resistance to stress. Although Hsp22 has been shown to have a chaperone-like activity *in vitro*, the mechanisms by which it extends lifespan *in vivo* are still unknown.

Genome-wide transcriptional analysis by microarray and comparative mitochondrial proteomic analysis by MALDI-TOF mass analysis have been performed to unveil differences in long-lived Hsp22 over-expressing flies and normal-lived control flies. Ubiquitous over-expression of Hsp22 using the GAL4/UAS system in *Drosophila* resulted in a ~ 30% increase in mean lifespan. The genomic analysis suggests that Hsp22 plays a role in lifespan determination by altering the regulation of the overall process of normal aging. Indeed, flies over-expressing Hsp22 display an up-regulation of genes normally down-regulated with age and involved in energy production, protein biosynthesis, protein turnover, and lipid metabolism. Mitochondrial proteomic analysis also supports a putative role of Hsp22 on lifespan determination by maintaining mitochondrial function and favoring proteolysis. The present data suggest the importance of the maintenance of protein homeostasis in aging and potential mechanisms of longevity in the Hsp22 over-expressing flies.

Preface

Although a large body of studies on the aging process has been performed for several decades, the mechanisms involved are complex and still highly debated. Many members of heat shock protein families (Hsps) have been implicated to influence the aging process in different organisms. The present study focusses on the mitochondrial chaperone Hsp22 in *Drosophila melanogaster*, and aims to unveil a detailed mechanism by which over-expression of Hsp22 prolongs longevity.

General introductions on the aging process and the heat shock protein families are described in Chapter 1. In Chapter 2, the longevity observed in flies over-expressing Hsp22 and several physiological features are compared with control flies. To define the differences in genome transcription and in proteome of Hsp22 over-expressing flies, we performed mitochondrial proteomic/transcriptomic comparisons. Chapter 3 validates the methodology of mitochondrial isolation and assessment of the purifity of the mitochondrial fraction. Chapter 4 describes a proteomic analysis of mitochondria and electron transport chain activity. Chapter 5 presents the transcriptomic analysis. On the basis of overall data obtained in this study, a potential mechanism of longevity in long-lived flies over-expressing Hsp22 is discussed in Chapter 6.

I would like to sincerely thank all the members of the jury for their effort to evaluate my Ph D. thesis.

Since September 2003, Dr. Robert, M. Tanguay has been the best supervisor for my Ph.D study. I have been impressed and encouraged a lot by his silent instruction. It was like a beacon light on the ocean. He printed out newly updated papers on aging study, kindly handwrited my name on top of the papers and layed them on my desk before my arriving in early morning. In addition, attending MiMage and LINK-AGE joint Summer School on Models and Methods in Ageing Research (Les Diablerets, Switzerland) last autumn was a great experience. I would like to thank my supervisor a lot for his thoughtful consideration.

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List of figures

List of tables

List of abbreviations

ABC	Adenosine triphosphate binding cassette
AD	Alzheimer' disease
ADP	Adenosine diphosphate
AGEs	Advanced glycation end products
ALS	Amyotrophic Lateral Sclerosis (Lou Gehrig's Disease)
ANOVA	Analysis of variance
ANT	Adenine nucleotide transporter (ADP/ATP translocase)
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
cDNA	Complementary deoxyribonucleic acid
CK2	Casein kinase II
CoQ	Ubiquinone coenzyme Q
DB	Decylubiquinone
DBH_2	Decylubiquinol (reduced decylubiquinone)
DCPIP	2,6-dichlorophenolindophenol
DmVDAC	*Drosophila* voltage-dependent anion-selective channel
DNA	Deoxyribonucleic acid
DNP	2,4-dinitrophenylhydrazone
DNPH	2,4-dinitrophenylhydrazine
dNTP	Deoxyribonucleotide triphosphate
DR	Dietary restriction
EAD	Early Alzheimer’s disease
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol tetraacetic acid
ERK	Extracellular signal-regulated kinase
EST	Expressed sequence tags
FADH	Flavin adenine dinucleotide (reduced form)
FOXO	Forkhead-family transctiption factor
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
GPX	Glutathione peroxidase
GR	Glutathione reductase
Grx	Glutaredoxin
GSH	Glutathione
GSTs	Glutathione S-transferases
GTP	Guanosine triphosphate
HCL	Hierarchical clustering
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HNE	4-hydroxy-2-nonenal
HSE	Heat shock element
HSF	Heat shock factor

HSPs	Heat shock proteins
HUVEC	Human umbilical vein endothelial cells
IEF/2D-PAGE	Isoelectric focusing two-dimensional polyacrylamide gel electrophoresis
IFMs	Indirect flight muscles
IGF	Insulin growth factor
IIS	Insulin/insulin growth factor-1 signaling
IMS	Mitochondrial intermembrane space
IP3	Inositol triphosphate
JNK	Jun N-terminal kinase
LC-MS/MS	Liquid chromatography with tandem mass spectrometry
LD_{50}	Median lethal dose (Lethal Dose, 50%)
LPO	Lipid peroxidation
MALDI-TOF	Matrix assisted laser desorption ionisation-time of flight
MAP	Mitogen-activated protein
MAPKAP	Mitogen-activated protein kinase activating protein
MCI	Mild cognitive impairment
MDA	Malondialdehyde
MIM	Mitochondrial inner membrane
MnSOD	Manganese superoxide dismutase
MOM	Mitochondrial outer membrane
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial deoxyribonucleic acid
NADH	Nicotinamide adenine dinucleotide (reduced form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NCLs	Neuronal ceroid lipofuscinoses
NF-κB	Nuclear factor-kappa B
NHE	Sodium-hydrogen exchanger (Na+/H+ exchanger)
NOS	Nitric oxide synthase
NP-40	Nonidet P-40 (nonyl phenoxylpolyethoxylethanol)
PBA	4-phenylbutyrate
Pi	Inorganic phosphate
PKC	Protein kinase C
polIII	RNA polymerase III
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
sHSP	Small heat shock proteins
SOD	Superoxide dismutase
16SrRNA	16S ribosomal ribonucleic acid
TAE	Tris-acetate-ethylenediaminetetraacetic acid

TCA	Tricarboxylic acid (citric acid)
TnH	Troponin-H
TOR	Target of rapamycin
Trx	Thioredoxin
TrxR	Thioredoxin reductase
UAS	Upstream activating sequence
VDAC	Voltage-dependent anion-selective channel

Contents

Chapter 1. General introduction

1.1 Theories of aging

The aging process is a complex phenomenon. This process is accompanied by a decreased capacity to tolerate and respond to various forms of stresses, and consequently has been associated with many diseases. Numerous aging theories have been proposed. Nevertheless, understanding the overall mechanisms related to aging is still unclear. Four main pathways have been shown to influence lifespan, the free radical theory (Harman, 1956), dietary restriction (McCay et al., 1935), the insulin/IGF-1 pathway (Clancy et al., 2001; Tatar et al., 2001), and the target of rapamycin (TOR) pathway (Kapahi et al. 2004).

1.1.1 Free radical theory

Harman proposed that aging, as well as the associated degenerative diseases, could be attributed to the deleterious effects of free radicals on various cell components (Harman, 1956). Although the concept for the generation of endogenous oxygen radicals was at first controversial, the later identification of superoxide dismutase (SOD), an enzyme which seems to be involved in the removal of superoxide anions, provided mechanistic support for Harman's hypothesis (McCord and Fridovich, 1969). Moreover, several evidences indicate that free radical mediated damages to cellular function contribute not only to aging, but also to cancer and multiple age-related neurological diseases including Alzheimer's disease (AD) (Ames, 1989; Markesbery et al., 2001; Markesbery and Lovell, 2006; Lovell and Markesbery, 2007a).

On the other hand, a relationship between the generation of age-inducing free radicals and the metabolic rate based on mitochondrial respiratory enzymes has been hypothesized. That is, the higher metabolic rate of an organism, the greater production of ROS and hence a shorter lifespan. However, a strict correlation between metabolic rate and lifespan has not been always observed. For instance, lowering ambient temperature in worms or flies slows the metabolic rate and then results in a concomitant extension of lifespan (Miquel et al., 1976). However, metabolic shift from fermentation to mitochondria-based aerobic respiration in yeast results in higher metabolic rate with increased oxygen consumption but leads to longer lifespan (Lin et al., 2002). Therefore, the relationship between oxygen consumption and ROS generation seems to be more complex than predicted. The relevant intracellular targets for ROS and the way it influences lifespan through oxidative modification are beginning to be understood.

1.1.2 Dietary restriction

The phenomenon of lifespan extension by dietary restriction (DR) was discovered in rats by Macay and colleagues (1935). The DR was achieved by feeding a reduced nutrient without starvation, and similar effects have been observed in various organisms such as yeast Saccharomyces cerevisiae (Jiang el al., 2000), fruitfly *Drosophila* (Partridge et al., 1987; Chippindale et al., 1993; Chapman and Partridge, 1996), mice (Weindruch et al., 1986; Bartke et al., 2001), dogs (Kealy et al., 2002), and rhesus monkeys (Lane et al., 2002 and 2004). Maximized longevity caused by DR, however resulted in reduced fertility (Holehan and Merry, 1986; Chippindale et al., 1993; Chapman and Partridge, 1996), thus the plausible mechanism for DR is that individuals may switch nutrient resources from reproductive functions to somatic maintenance under conditions of food scarcity. Therefore, they can survive more effectively by successfully postponing reproduction instead of making a futile attempt for reproduction (Holliday, 1989; Masoro and Austad, 1996).

On the other hand, there is evidence that DR causes increased resistance against environmental stressors but no decrease of metabolic rate in the nematode Caenorhabditis elegans (Houthoofd and Vanfleteren, 2006). The lifespan extension by metabolic stability may offer another plausible explanation of the longevity effect of DR. However, although the almost universal extension of lifespan by DR is suggestive of an evolutionary conservation, the molecular mechanisms by which DR extends lifespan have not been fully revealed in any organism (Partridge et al., 2005). Therefore, it remains unproven whether the extension of lifespan by DR is accomplished by similar mechanisms in different species.

1.1.3 Insulin/insulin growth factor (IGF)-like signaling pathway

It has been discovered in Caenorhabditis elegans that reduction of insulin/IGF-1 signaling (IIS) by using a single gene mutation could result in a large increase of adult lifespan (Friedman and Johnson, 1988; Kenyon et al., 1993; Kimura et al., 1997). The mutant of age-1 gene increased 50% of average lifespan in Caenorhabditis elegans (Friedman and Johnson, 1988; Johnson, 1990), and daf-2 mutations increased lifespan by more than 100% (Kenyon et al., 1993). Also, the lifespan extension required the forkhead-family transcription (FOXO) factor DAF-16 activity (Kenyon et al., 1993; Dorman et al., 1995; Larsen et al., 1995; Lin et al., 1997; Ogg et al., 1997). Loss-of-function mutations in daf-16 gene suppressed the lifespan extension of daf-2 and age-1 mutants (Dorman et al., 1995). This finding has been recently extended to fruit flies

and rodents, demonstrating that the role of insulin/IGF-1 signaling in the control of lifespan is evolutionarily conserved (Partridge and Gems, 2002; Tatar et al., 2003). For instance, a number of long-lived mutant mice have appeared to modulate the insulin/IGF-1 pathway in an analogous manner to the daf-2/age-1/daf-16 mutants in Caenorhabditis elegans (Balaban et al., 2005), such as displaying reduced activities of DAF-2, an insulin/IGF-1-like receptor (Kimura et al. 1997), AGE-1, a PI 3 kinase (Morris et al. 1996), or downstream signaling components (Guarente and Kenyon, 2000). These mutant mice are smaller than their wild type counterparts, with their dwarf status as a result of deficiencies in several secreted factors including growth hormone, thyroid hormone, and often IGF-1 (Quarrie and Riabowol, 2004).

1.1.4 Target of rapamycin (TOR) signalling pathway

The TOR kinase is part of a putative amino acid sensing pathway, which controls cell growth by altering gene transcription and translation (Oldham and Hafen, 2003; Hay and Sonenberg, 2004). When amino acid levels are high, TOR up-regulates translation through the activation of ribosomal S6 kinase (S6K). On the contrary, when amino acid levels are low, the reduced TOR signaling leads to up-regulation of autophagy and protein degradation/turnover within lysosomal autophagosomes. In *Drosophila*, reduction of TOR signaling increases lifespan, and it has been suggested that the effects of DR on lifespan result from reduced TOR signaling (Kapahi et al., 2004).

1.2 Genes and proteins influencing aging process

1.2.1 Transcriptome analysis of aging in biological systems

Genetic approaches through transcriptomic analysis or gene mutation to dissect the molecular mechanism on aging and longevity determination have indicated that several specific genes are involved in the regulation of the aging process and longevity. In *Drosophila* melanogaster, the longevity genes identified by gain- or loss- of function mutations are Mth encoding a putative G-protein coupled receptor (Lin et al., 1998), Indy encoding a sodium dicarboxylate cotransporter of tricarboxylic acid cycle (TCA) intermediates (Rogina et al. 2000), chico encoding insulin receptor substrate (Clancy et al., 2001), and InR encoding the insulin-like receptor (Tatar et al., 2001). In addition, an extended longevity has been observed in transgenic flies over-expressing Cu/Zn superoxide dismutase and catalase (Orr and Sohal, 1994), Cu/Zn SOD only (Sun and Tower, 1999), or a molecular chaperone, Hsp70 (Tatar et al. 1997). On the other hand, it has been very recently demonstrated through transcriptional profiling by microarray analysis, that MnSOD over-expressing transgenic flies showed lifespan extension by specifically modulating genes involved in carbohydrate metabolism (glycolytic enzymes) and mitochondrial electron transport chain (cytochrome-c oxidase subunit Va, NADH dehydrogenase) (Curtis et al., 2007).

In *C. elegans*, the reduction of gene expression of the homologue of drIndy by RNAi resulted in an increased lifespan. Suppressed expressions of nac-2 and nac-3, Na+-dependent transport genes, increased the mean lifespan but not nac-1 (Fei et al., 2003 and 2004). In addition, analysis of the transcriptional response to reduced IIS-target transcription factor FOXO/DAF-16 has identified genes that are up-regulated, including MnSOD (Honda and Honda, 2002) and hsp16 (Murphy et al., 2003; McElwee et al., 2003), as well as genes that are down-regulated such as insulin-like peptides gene (ins-7) and guanylyl cyclase (gcy-18) (Murphy et al., 2003).

Moreover, cross-species comparisons of genome-wide expression patterns during aging have presented the general or species-specific signatures of aging (McCarroll et al., 2004; Smith et al., 2007). Very recently, Curtis and colleague compared the gene expression profiles resulting from MnSOD over-expression in *Drosophila* with those of genes regulated by daf-2/daf-16 in C. elegans, and reported that part of lifespan regulation by the insulin/insulin growth factor

(IGF)-like signaling (IIS) may proceed through MnSOD being likely a general effector of longevity (Curtis et al., 2007).

1.2.2 Proteome analysis of aging in biological systems

According to many reports, an altered expression of proteins has been detected during the aging process: such as heat shock proteins (HSPs) (Blake et al., 1991; Snoeckx et al., 2001), antioxidative enzymes (Cand and Verdetti, 1989; Junqueira et al., 2004), superoxide dismutase (SOD) (Landis and Tower, 2005), nitric oxide synthase (NOS) (Lyons et al., 1997; Chou et al., 1998; Vasa et al., 2000; Zieman et al., 2001), sodium-hydrogen exchanger (NHE) (Taylor and Starnes, 2003), mitogen-activated protein (MAP) kinases (Izumi et al., 1998; Suh, 2002; Gabai and Sherman, 2002), Jun N-terminal kinase (JNK) (Suh, 2002), extracellular signal-regulated kinase (ERK) (Suh, 2002) among others.

Recently, the mitochondrial proteome has been often implicated in aging and longevity issues specially on finding the relationship between mitochondrial energy metabolism and the aging process. Groebe et al., (2007) identified ATP synthase as a target affected by age-dependent processes, through analysis of the comparative mitochondrial proteomes in various species. Moreover, the comparative proteomic analysis of aging monkey hearts presented gender-specific alteration in key enzymes such as those of glycolysis, glucose oxidation, TCA cycle, and electron transport chain (complexes III-V), which show age-dependent decrease in males but not in females (Yan et al., 2004).

1.3 Heat shock proteins and functions

The heat shock proteins (HSPs) were first discovered by a specific pattern of polytene chromosomal puffing induced by an accidental thermal shock in the salivary glands of *Drosophila* (Ritossa, 1962). The HSPs are highly conserved in various species, and can be assigned to several families on the basis of sequence homology of their encoding genes and typical molecular weight of HSPs: HSP110, HSP100, HSP90, HSP70, HSP60, and small HSPs (sHSPs) families (Schmitt and Langer, 1997). An early known function of the HSPs was their involvement in homeostasis and an association with resistance to heat shock and other stresses. However, it has been additionally discovered that certain HSPs have a chaperone function involved in modifying the structure of proteins. HSPs acting as molecular chaperones, play essential roles in protein folding, trafficking, assembly and degradation. Thus, upon stress, the HSPs prevent protein misfolding or aggregation, thereby maintaining the critical cellular structures and functions and protecting against apoptotic or necrotic cell death. Further description for chaperoning function of HSPs is in 1.3.2.

1.3.1 Regulation of heat shock response

The expression of HSPs is regulated by multiple mechanisms, among which the transcriptional regulation is most prominent. Although the structure and function of HSPs are remarkably conserved throughout evolution, the transcriptional regulatory mechanisms are conferred to quite different systems in prokaryotes and eukaryotes.

1.3.1.1 Prokaryotic machinery for transcriptional regulation of heat shock response

The prokaryotic HSPs, characterized in Escherichia coli include DnaK (prokaryotic Hsp70), DnaJ (Hsp40), GrpE, GroEL (Hsp60), and GroES (Hsp10). Regulation of these bacterial heat shock proteins is exerted by RpoH (i.e. stress inducible σ32 subunit of RNA polymerase) heat shock factors (Grossman et al., 1987; Arsène et al., 2000). Upon exposure to stress stimuli including heat shock, the concentration of σ32 is greatly increased through enhanced synthesis and increased stability, resulting in preferred transcription of σ32-dependent heat shock genes (Straus et al., 1987; Yura and Nakahigashi, 1999; Arsène et al., 2000). Hence, the σ32-mediated transcription is controlled by a negative feedback system. As an example, the accumulating DnaK-DnaJ-GrpE chaperone machinery binds to σ32 and inhibits its activity

(Fig. 1-1) (Tilly et al., 1983; Straus et al., 1990; Tomoyasu et al., 1998). Moreover, binding of DnaK-DnaJ to the σ32 promotes its degradation by the ATP-dependent metalloprotease FtsH (Tatsuta et al., 1998). Therefore, availability of DnaK-DnaJ is a direct sensor of cellular stress and a regulator of heat shock transcription (Tomoyasu et al., 1998).

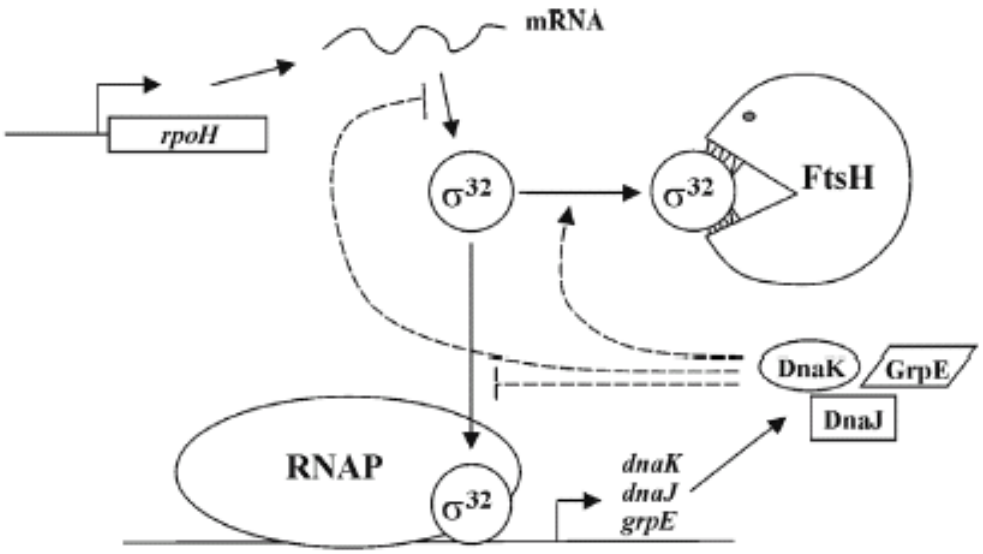


Fig. 1-1. Regulatory mechanisms of the prokaryotic heat shock response
In *E.coli*, the heat shock response is regulated by the σ32 factor. Accumulation of the heat shock proteins DnaK, DnaJ, and GrpE provides a negative feedback mechanism by inhibiting σ32 translation and association with the core RNA polymerase and by promoting σ32 degradation by FtsH. (Reference: Hietakangas and Sistonen, 2006)

1.3.1.2 Eukaryotic system for transcriptional regulation of heat shock response

The transcriptional regulation of heat shock response in eukaryotes is well conserved and regulated more systemically. As a component involved in the transcriptional process, heat shock element (HSE), a specific DNA sequence responsible for the transcriptional activation of heat shock genes, was identified within their promoters in *Drosophila* (Pelham, 1982 and 1985). The consensus HSE consists of contiguous inverted pentameric repeats of the sequence nGAAn (Fig. 1-2) (Amin et al., 1988; Xiao and Lis, 1988; Perisic et al., 1989; Kroeger and Morimoto, 1994). More precise comparison of the HSEs in various organisms reveals that yeast

and *Drosophila* HSEs are composed primarily of consensus sites (5'-nGAAn-3') (see Fig. 1-2) (Amin et al., 1988; Xiao and Lis, 1988). In larger eukaryotes only the G residue remains highly conserved, and there are variant sequences such as, nGGGn and nGACn in the HSP70 HSE (Abravaya et al., 1991; Cunniff et al., 1991; Gallo et al., 1991).

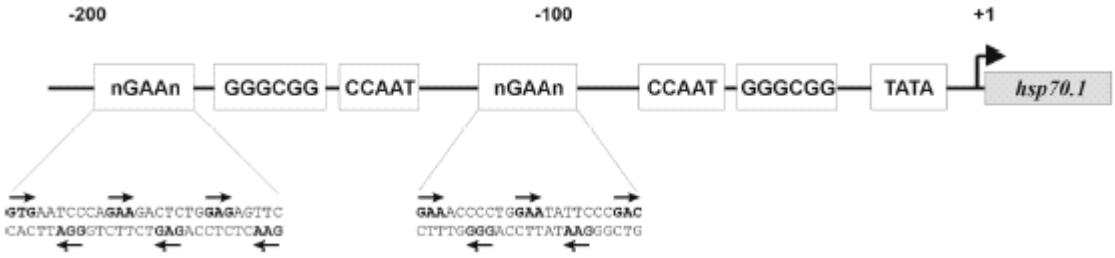


Fig. 1-2. Example of HSEs within human hsp70.1 promoter site

The human *hsp70.1* promoter contains a proximal and distal HSE with five and six inverted nGAAn repeats. In addition, GC-, CCAAT-, and TATA-boxes exist for other transcriptional factor binding and constitutive *hsp70.1* expression, and are involved in the maintenance of chromatin accessibility. (Reference: Hietakangas and Sistonen, 2006)

Furthermore, the heat shock factor (HSF) that binds to the HSE and activates HSP gene transcription was identified (Parker and Topol, 1984; Topol et al., 1985) and it has been available to elucidate the mechanism of regulating HSP expression with HSE. There are different members of the HSF family that share homologous functional domains. In the case of yeast and fruit fly, a single HSF is responsible for HSE-mediated transcription (Sorger and Pelham, 1988; Wiederrecht et al., 1988; Clos et al., 1990). In vertebrates, however, several members of HSF families exist sharing functional and structural properties, such as domains involved in DNA binding and oligomerization (Fig. 1-3). In the case of mammals, three HSF family members of HSF1, HFS2, and HSF4 have been found, and HSF1, HSF2 and HSF3 have been seen in avian species (Rabindran et al., 1991; Sarge et al., 1991; Schuetz et al., 1991; Nakai and Morimoto, 1993; Nakai et al., 1997).

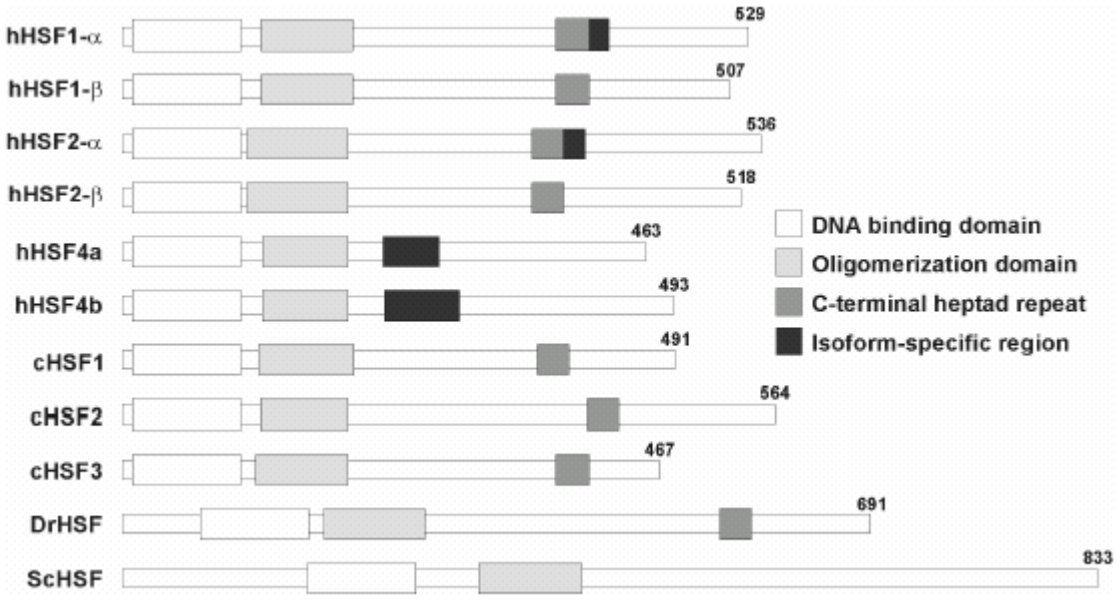


Fig. 1-3. Schematic analysis for conserved domains of heat shock transcriptional factors among various organisms

Abbreviation: h (human), c (chicken), Dr (fruit fly), Sc (budding yeast). All HSFs have a DNA-binding domain and oligomerization domain, which is involved in trimerization of HSF monomer. HSF4 and ScHSF lack the C-terminal heptad repeat, which negatively regulates trimerization. Mammalian HSF1, HSF2, and HSF4 are expressed as two alternatively spliced isoforms. (Reference: Hietakangas and Sistonen, 2006)

However, not all heat shock factors are stress responsive. In addition to the stress inducible activation, some members of the HSF family are also activated under non-stressful conditions. Among the vertebrate HSFs, only HSF1 and the avian-specific HSF3 have been seen to be activated by stress stimuli and to be essential for the transcriptional regulation of the heat shock response (Nakai et al., 1995; McMillan et al., 1998; Tanabe et al., 1998; Xiao et al., 1999). Therefore, the HSFs that are refractory to stress stimuli would regulate transcription under other circumstances, such as during development and differentiation, and possibly have target genes distinct from the classical heat shock genes (Pirkkala et al., 2001). Specific sequence in the HSF protein can cause the differentiated physiological function and characteristic property, while the similarity within HSF sequence provides a prediction for the relationship between HSF families as shown in Fig. 1-4.

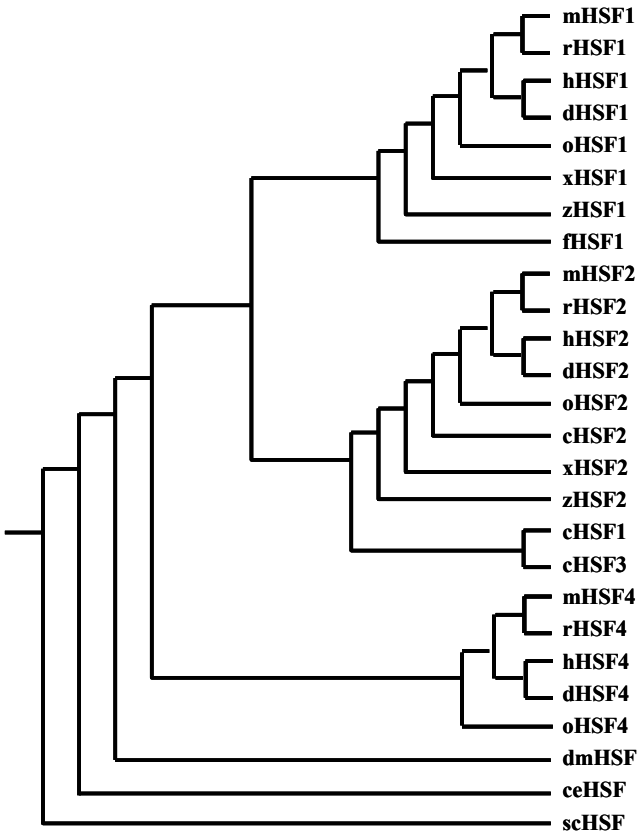


Fig. 1-4. Evolutionary relationships of HSF family

Abbreviations: m (mouse), h (human), r (rat), d (dog), o (opossum), c (chicken), x (Xenopus), Z (zebrafish), f (fugu), dm (Drosophila), cd (C. elegans), sc (S. cerevisiae). (Reference: Orosz and Benjamin, 2007)

On the other hand, several studies on the activation of HSF have reported that HSF/HSF1 is activated by a multi-step process, where the induction of DNA binding and the increase in transcriptional activity can be distinct (Wu, 1984; Mosser et al., 1988; Gallo et al., 1991; Sarge et al., 1993; Westwood and Wu, 1993; Holmberg et al., 2002). While most general transcription factors are activated through dimerization, HSF1 is activated by a transition from monomer to trimer, which causes a 104-fold increase in HSE-binding activity (Wu, 1995). An exception to this rule is the budding yeast HSF that has been indicated to exist constitutively as a trimer capable of binding to the HSEs even under non-stressful conditions (Sorger et al., 1987; Jakobsen and Pelham, 1988). HSF/DNA binding occurs very rapidly and is recruited to hsp gene promoter within seconds following heat shock, and the levels of HSF are saturated on the promoter in less than two minutes (Boehm et al., 2003). It has also been reported that the

process of HSF oligomerization can be affected by cysteine residue dependent- (Ahn and Thiele, 2003) or redox sensitive manner (Goodson and Sarge, 1995; Zhong et al., 1998, Ahn and Thiele, 2003) and thus possibly regulated post-translationally.

1.3.1.3 Post-translational regulation of heat shock response

Transcriptionally active HSF was shown to be phosphorylated in yeast, *Drosophila* and mammalian cells, which indicates the possible role of phosphorylation events in regulating the heat shock response. Indeed, it has been clarified that the activity of HSFs is under post-translational control, requiring the serial events of trimerization, DNA binding, and hyperphosphorylation. However, whether the role of phosphorylation of HSF is in activating or deactivating still remains elusive, and the critical residues for constitutive phosphorylation of HSF have not been precisely determined in the whole molecule except that there is a predominant phosphorylation in serine residues (Knauf et al., 1996; Kline and Morimoto, 1997). Recently, analysis of the numerous HSF target genes through a genome-wide analysis, especially concerning the chaperone function in the heat shock response, has indicated the involvement of HSF regulation not only in the heat shock response, but also in proteolysis, vesicular and small molecular transport, cell wall and cytoskeleton maintenance and finally carbohydrate and energy metabolism (Hahn et al., 2004). Therefore, the phosphorylation of HSF is likely to be regulated in a target gene-specific manner (Hashikawa and Sakurai, 2004). Furthermore, the interplay between different HSF family members and other interacting proteins involved in signaling pathways, such as apoptotic cell death or survival program, brings further complexity to HSF-mediated transcription (Bijur and Jope, 2000; Dai et al., 2000; He et al., 1998; Xavier et al., 2000).

In addition to phosphorylation, Hong et al. (2001) indicated that HSF1 undergoes stress-inducible SUMO modification to acquire HSF1 DNA-binding activity. The regulatory roles of sumoylation and phosphorylation are still enigmatic, and their concurrent regulation of HSF1 activation is likely to be essential for the fine-tuning of heat shock transcription in response to specific stimuli. Possibly, the cytoplasmic-nuclear localization of HSF may also address the post-translational regulatory step during heat shock (Kiang and Tsokos, 1998). In *Drosophila* and *Xenopus*, HSF is predominantly localized in the nucleus before and after heat shock, while the cytoplasmic-nuclear relocalization event of HSF may play considerable role in the

regulation of stress response in human or mouse tissue culture cells (Westwood et al., 1991; Zuo et al., 1995; Orosz et al., 1996; Tanguay and Wu, 2006) as indicated in Fig. 1-5.

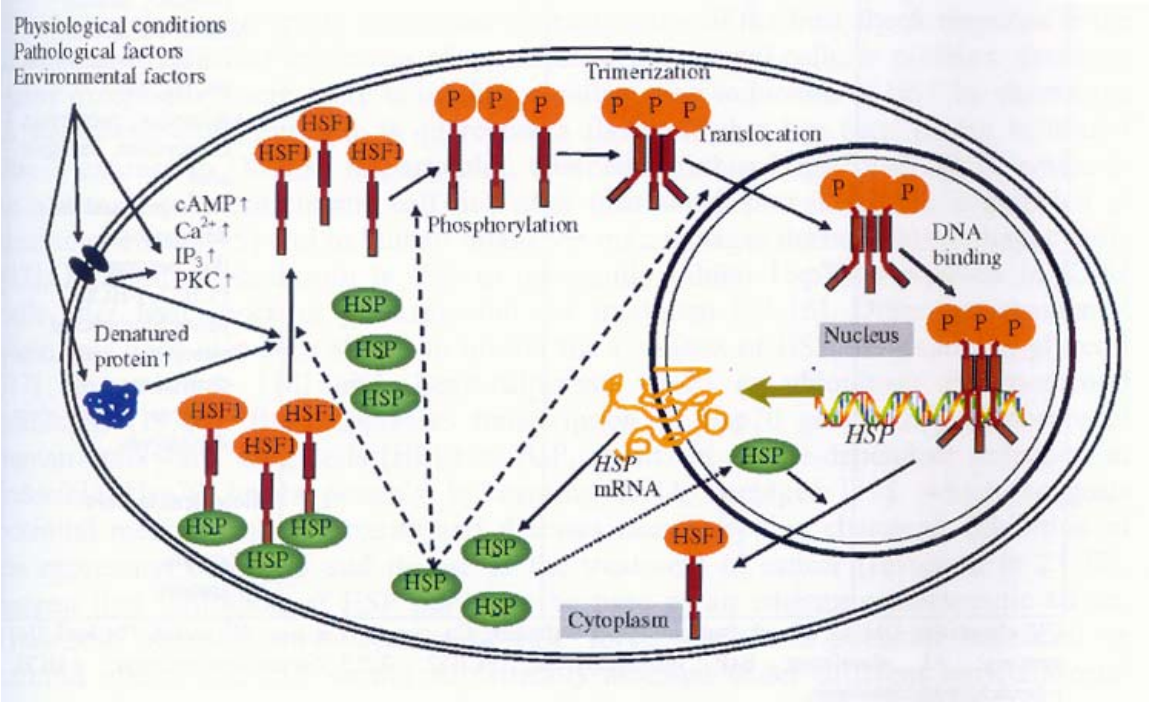


Fig. 1-5. Proposed mechanism of regulation of the heat shock response

Extracelllar and intracellular stresses act on cellular membranes or induce changes of intracellular components such as denatured proteins. Under normal conditions, HSF1 is kept inactive through binding with HSP and other regulatory proteins. Upon stress, HSF1 is released from this complex and activated through phosphorylation, trimerization and nuclear translocation. Newly translated HSP can then achieve their role in their respective intracellular compartments and regulate the level of HSF1 activation at different steps of the process. cAMP, cyclic AMP; IP_3, inositol triphosphate; PKC, protein kinase C. (Reference: Tanguay and Wu, 2006)

1.3.2 Hsps and chaperone

Since Laskey and colleagues began to use the name of 'molecular chaperone' in 1978, more than 50 families of molecular chaperones have been found (Ellis, 2005). The molecular chaperones are involved in many essential cellular functions, such as metabolism, growth, differentiation and programmed cell death, and protein degradation by the proteasome machinery, all of which are carried out through protein assembly and transport (Schmitt and Langer, 1997; Ranford et al., 2000). Most of the chaperones are known to use ATPase cycles to drive non-native protein binding for their active role in folding or unfolding (Bukau and Horwich, 1998; Frydman, 2001). Many heat shock protein families such as Hsp100/110, Hsp90, Hsp70, Hsp60/Hsp10 (chaperonins), and the small Hsp (sHsp) families, present a chaperone (chaperone-like) function.

Table 1-1. Diversity of the major molecular chaperone families

Family	Chaperone functions
Chaperonin (cpn10, hsp10, co-chaperonin, early pregnancy factor, GroES)	'Co-chaperonin' to Chaperonin 60; promotes folding of substrates that are bound to Chaperonin 60
Small heat shock proteins (sHsp)	Diverse class of proteins (molecular weight ranged 15-43kDa); bind non-native proteins; ATP independent chaperone function in vitro; but, αB-crystallin ATP binding induces chaperone-like functions
Hsp40 (DnaJ-like)	Co-chaperones that regulate the activity of Hsp70; some can bind non-native proteins themselves
Hsp60 (Chaperonin 60; cpn60, hsp60, hsp65, GroEL)	ATP-dependent folding and/or refolding of ~15-30% of total cellular proteins
Hsp70 (DnaK)	Prevent the aggregation of unfolded polypeptides; disassemble multimeric protein complexes; involved in protein trafficking; regulate the heat-shock response
Hsp90	Specific functions in regulating signal transduction pathways, through their actions on certain kinases and steroid receptors; also general chaperone activity
Hsp100 (Clp)	Disassemble protein oligomers and aggregates
Hsp110	High degree of homology with the Hsp70 family; little known about functions

(Modified table from Ranfold et al., 2000)

1.3.2.1 Hsp60 chaperonin system

The chaperone member, GroEL-GroES is in the class of Hsp60 molecular chaperone (chaperonin 60), which was first found in temperature-sensitive (ts) mutants of the essential *groE* operon in *E. coli* (Georgopoulos et al., 1973; Georgopoulos and Hohn, 1978; Chandrasekhar et al., 1986). GroEL-GroES prevents aggregation of native proteins by encapsulating each partly folded chain inside its cage structure, where the chain can continue to fold by sequestering the proteins from similar chains (Saibil and Ranson, 2002; Fenton and Horwich, 2003; Ellis, 2006). (Fig. 1-6)

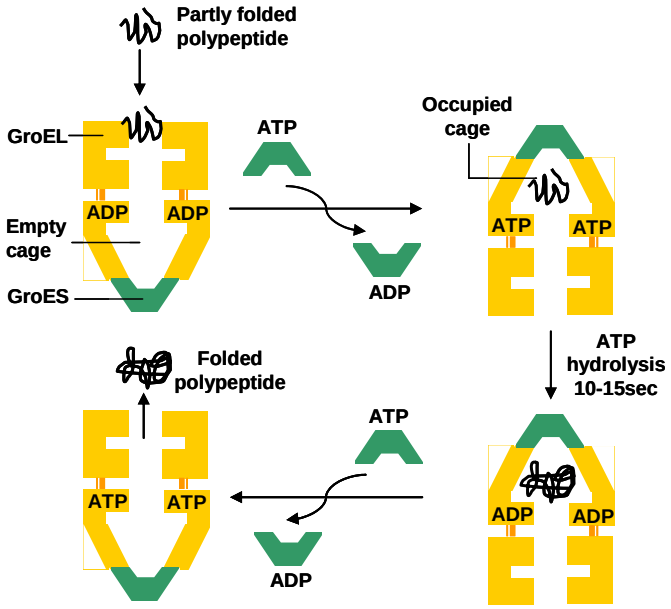


Fig. 1-6. Schematic description of GroEL-GroES chaperonin system

GroEL consists of two rings, each made of seven ATPase subunits arranged back to back, whereas GroES consists of a single dome-shaped ring of seven smaller subunits. The nucleotide-binding abilities of the two GroEL rings are mutually exclusive. In the presence of ATP or ADP, GroES binds to the same ring that binds the nucleotide, creating a cage at that end. The binding of GroES and ATP triggers release of the polypeptide chain into the newly created cage. It takes 10-15 seconds for the ATP to be hydrolysed to ADP by the ring that contains the folding protein. (Reference; Ellis, 2006)

1.3.2.2 Hsp70 and Hsp90 chaperones

Molecular chaperones of the Hsp70 and Hsp90 families are the major components of the cytosolic protein folding and protein quality control system (Frydman 2001; Wegele et al., 2004), but they seem to prefer different types of substrates. The Hsp70 chaperones mainly assist newly synthesized proteins in attaining their native structure and help to refold partly denatured and even aggregated proteins. The function of Hsp70 chaperones is based on their ability to interact transiently with short peptide stretches of substrate proteins in an ATP-dependent manner. Three distinct domains of the Hsp70 family are composed of N-terminal ATPase domain, a central peptide-binding domain, and a C-terminal domain (Flaherty et al., 1990; Zhu et al., 1996). The substrate-binding domain is formed by a hydrophobic pocket of two sheets of ß-strands (Zhu et al., 1996), and binds to patches of hydrophobic amino acids of folding intermediates or misfolded proteins (Mayer et al., 2000; Rüdiger et al., 2001). The C-terminal domain functions as a lid that permits entry and release of polypeptide substrates (Bukau and Horwich 1998; Zhu et al., 1996). The opening and closing of the lid seems to be regulated by ATP binding and hydrolysis by the ATPase domain. The peptide-binding domain possesses a low affinity for substrate proteins in the ATP-bound state, whereas the Hsp70 chaperone undergoes conformational changes that lead to a strong binding of the substrate molecule upon ATP hydrolysis (McCarty et al., 1995; Theyssen et al., 1996). ATP hydrolysis is enhanced by Hsp40 co-chaperones, and the substrate protein is released when the bound ADP is exchanged to ATP by nucleotide exchange factors such as BAG-1 or BAG-1 related proteins.

Hsp90 also promotes protein folding as one of the most abundant proteins under unstressed condition representing at least 1% of the total soluble cytosolic protein (Lai et al., 1984). It prevents unfolding and aggregation of already folded proteins, thus serves as a “house-keeping” function (Picard, 2002). The Hsp90 chaperones keep meta-stable signal proteins such as steroid hormone receptors in a conformation that possess a high affinity for their substrates, and its binding of a hormone molecule stabilizes the substrate protein. The small acidic protein p23 (Sba1p in yeast), co-chaperone of Hsp90 interacts with the N-terminal domain of Hsp90, and the interaction seems to stabilize substrate binding in an Hsp90-p23 complex (Dittmar and Pratt, 1997). Furthermore, in the case of the co-chaperone Hop, more promiscuous interaction specificity is exhibited to bind to Hsp70 and Hsp90, and thus there seems to exist a linkage between Hsp70 and Hsp90 chaperones by the coupling factor (Perdew and Whitelaw, 1991; Wegele et al., 2003 & 2004).

1.3.2.3 sHsp as chaperones

Small heat shock proteins (sHsp) are characterized by a relatively low monomeric molecular mass (15-43kDa) and a conserved α-crystallin domain of 100 amino acid residues displaying a sequence similarity to the vertebrate eye lens protein α-crystallin which prevents protein precipitation and cataract formation in the eye lens (Corces et al., 1980; Ingolia and Craig, 1982; Russnak and Candido, 1985). The small Hsps are ubiquitous proteins which consist of three domains including an α-crystallin domain, a N-termical hydrophobic region and a short C-terminal extension (Narberhaus, 2002; Laksanalamai and Robb, 2004), and they can be located in the cytoplasm, nucleus, mitochondria, endoplasmic reticulum and chloroplast in higher plants (Boston et al., 1996; Michaud et al., 2002).

The various functions of sHsps are regulated by their phosphorylation state. For example, as shown in Fig. 1-7, human Hsp27 may be phosphorylated on residues Ser15, Ser78, and Ser82 (Landry et al, 1992; Stokoe et al., 1992) while murineHsp27 may be phosphorylated on Ser15 and Ser86 (Gaestel et al., 1991). Phosphorylation of mammalian Hsp27 is mediated by the kinase MAPK-activating protein (MAPKAP) 2/3, a substrate of p38 kinase, and also by protein kinase C (Maizels et al., 1998). Chaperones are often regulated by cofactors or ATP, it is still debated whether ATP binding and hydrolysis are involved in functional regulation or not, or if other mechanisms are observed in the case of sHsps (Halsbeck, 2002). Unlike most other chaperones including Hsp100 and Hsp70, the activity of sHsps in vitro has so far been found to be independent of ATP binding and hydrolysis (Haslbeck et al., 1999; Van Montfort et al., 2001a; Liberek et al., 2008). In the case of αB-crystallin, however, ATP binding leads to structural arrangement and exposure of hydrophobic residues (Muchowski and Clark, 1998; Muchowski et al., 1999).

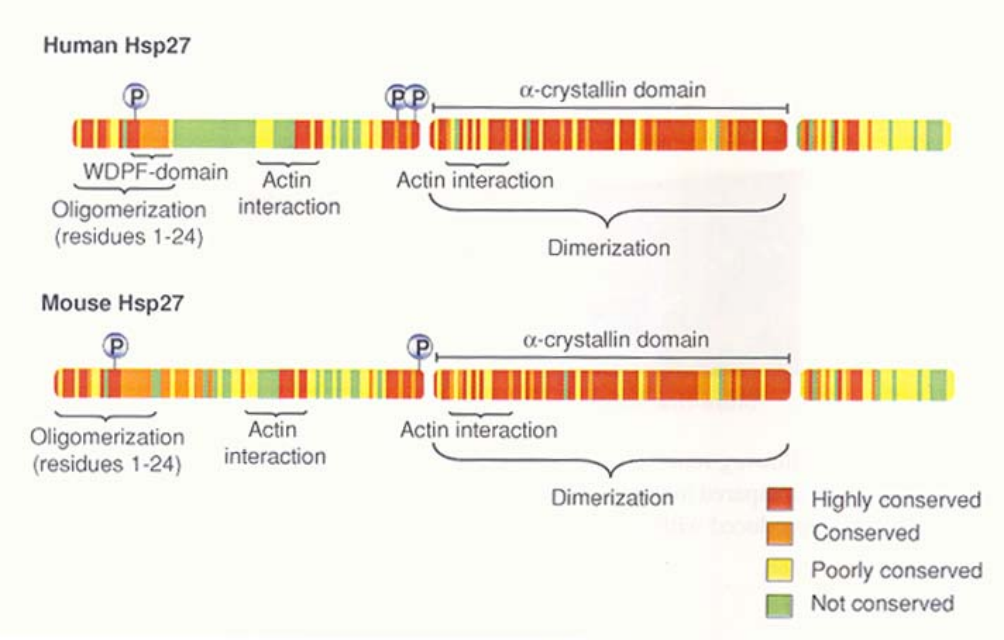


Fig. 1-7. Schematic representation of human and mouse Hsp27 protein

Phosphorylatable residues are indicated by the letter P above the amino acid sequence. (Reference: Orejuela et al., 2007)

Deletion of sHsps genes does not cause a thermo-sensitivity, while overproduction of sHsps increases thermo-tolerance in a number of organisms and cell types, suggesting the involvement of sHsp in the control of aggregation and disaggregation upon heat shock (Kitagawa et al., 2000; Nakamoto et al., 2000). Several in vivo studies showed that sHsps in stressed cells localized mostly in an insoluble fraction (Allen et al., 1992; Laskowska et al., 1996; Basha et al., 2004), and not only the ability to bind aggregates but also a limited ability to protect enzymes from heat denaturation were reported in in vitro studies (Jakob et al. 1993; Studer and Narberhaus, 2000). The sHsps form an oligomeric structure, which constantly exchange subunits, presumably dimers, and the increase in temperature destabilizes proteins, resulting in aggregation-prone folding intermediates that tend to associate with the temperature-activated sHsps (Haslbeck, 2002; Haslbeck et al., 2005). For the activation of sHsps chaperone function, not only the temperature-sensing system, but also additional signals such as phosphorylation seem to be necessary but not sufficient for dissociation of sHsp oligomers (Haslbeck, 2002).

Table 1-2. Best representative members of the sHsp families, their quaternary structure and their chaperone activity

	Protein	Organisms	Structure	Chaperone activity
Archaea	Hsp16.5	*M. jannaschii*	24mer	Yes
Prokaryotes	IbpA/IbpB	*E. coli*	oligomer	Yes
	Hsp16.3	*M. tuberculosis*	9mer	Yes
Eukaryotes				
Yeast	Hsp26	*S.cerevisiae*	24mer	Yes
	Hsp42	*S.cerevisiae*	oligomer	Yes
Invertebrates	Hsp27	*D. melanogaster*	-	Yes
	Hsp26	*D. melanogaster*	-	Yes
	Hsp23	*D. melanogaster*	-	Yes
	Hsp22	*D. melanogaster*	-	Yes
	Hsp16.2	*C. elegans*	-	Yes
	Hsp12.2/12.3	*C. elegans*	4mer	No
Vertebrates	HspB1, (Hsp25/Hsp27)	mammal	16-32mer /oligomer	Yes
	HspB2/HspB3	mammal	oligomer	-
	HspB4/HspB5, (αA-/αB-crystallin)	All vertebrates	32mer	Yes
	HspB6, (Hsp20/p20)	mammal	oligomer	No
	HspB8, (Hsp22/H11kinase)	mammal	monomer	Yes
Plant	Hsp18.1	*Pea (P. sativum)*	oligomer	Yes
	Hsp22	*Pea (P. sativum)*	-	-
	Hsp21	*A. thaliana*	oligomer	Yes
	Hsp16.9/Hsp17.8	*Wheat (T. aestivum)*	12mer	Yes
	Hsp17.3/Hsp17.7	*Tomato (L. peruvianum)*	-	Yes

1.3.3 Hsps and aging

Upon aging and longevity, the expression of Hsp chaperones has been reported in various organisms. Unbalanced chaperone requirement and/or chaperone capacity in aged organisms causes the accumulation of aggregated proteins, which often results in folding diseases, mostly of the nervous system, due to the limited proliferation of neurons. Therefore, over-expression of chaperones often delays the onset or diminishes the symptoms of the disease (Sőti and Csermely, 2002b), and increased chaperone induction can lead to increased longevity (Tatar et al., 1997; Morrow et al., 2006).

During aging in *Drosophila*, Hsp70 protein was induced 7- to 10-fold in thorax of old flies (36-38 day), relative to young flies (5-7 day) with the absence of heat shock, while no increase was observed in head or abdomen (Wheeler et al.,1995). In addition, RNA increase of hsp22 and hsp23 genes was observed in the thorax at the later time points (approximately after 30 day), the increased expression was 8- to 10- fold for hsp22 and 4- to 8-fold for hsp23 between days 20 and 40 (Wheeler et al.,1995). However, interestingly, most heat shock genes (hsp22, hsp23, hsp26, hsp27, all hscs+hsp70) in the head or abdomen exhibited an accumulation of RNA at day 2 after eclosion, which then decreased with age (Wheeler et al., 1995). Microdensitometric determinations of the low molecular weight heat shock polypeptides on [35S] methionine-labeled autoradiographs of five age groups (5 days, 16 days, 26 days, 47 days, and 54 days) revealed that their maximum expression occurs at 47 days for a population of flies with a mean lifespan of 33.7 days (Fleming et al., 1988). Marin et al. (1993) also presented a distinct tissue-specific cellular localization and age-dependent decrease of Hsp23 (in central neuropile of brain and thoracic ganglion) and Hsp26 (in cytoplasm of spermatocytes and spermatid bundles in testis) expression. It has been shown that 2- to 10-fold higher hsp22 RNA levels correlate with an increased lifespan in fly lines genetically selected for increased stress resistance (Kurapati et al., 2000). Over-expressing Hsp22 also has a beneficial effect on the longevity of flies and on their resistance to oxidative stress (Morrow et al., 2004a & 2004b).

On the other hand, given the role of HSF1 as a major regulator of cellular stress resistance and the fact that Hsp gene expression is poorly induced in aged cells and animals, it comes as no surprise that HSF1 regulates longevity in C. elegans (Garigan et al., 2002; Hsu et al., 2003; Morley and Morimoto 2004). It was found that reducing HSF1 activity accelerates tissue aging and shortens lifespan, while the over-expression of HSF1 extends lifespan (Hsu et al., 2003). In

addition, Walker and Lithgow (2003) reported that the introduction of extra copies of hsp16 gene confers stress resistance and longevity both in a wildtype and a long-lived mutant strain of C. elegans. It was demonstrated that lifespan is determined in part by insulin-like regulation through DAF16 transcription factor being essential for the maximal expression of molecular chaperones hsp16. Although HSF1 and DAF16 are known to function independently of each other, they appear to share a subset of target genes such as small heat shock proteins, whose expression is impaired upon inactivation of DAF16 or HSF1 (Hsu et al., 2003).

The relationship between aging (longevity) and mammalian Hsp chaperones can be studied either at the protein level measuring the age-dependent basal or induced levels of Hsps, or at the transcript level studying the association of polymorphisms of Hsp genes involved in longevity and searching for an aging-dependent particular allele, genotype, or haplotype that co-segregates (Singh et al., 2007). Otherwise, an age-related variation in the ability to respond to stress, which can be measured by the inducibility of Hsps following heat shock at a certain temperature, has been used in mammalian cellular systems. Pahlavani et al., (1995) reported that the splenic lymphocytes isolated from old (24-26 months) rats expressed a marked decrease in the induction of Hsp70 protein levels after heat treatment when compared with young (4-5 months) rats and obtained the same result in peripheral lymphocytes isolated from rhesus monkeys. Consequently, it was revealed that hsp70 gene expression is attenuated due to cell aging in tissue culture models utilizing fibroblasts and lymphocytes isolated from human subjects (Deguchi et al., 1988; Liu et al., 1989; Liu et al., 1991; Luce and Cristofalo, 1992; Jurivich et al., 1997). Various cell types isolated from aging rodents have shown similar attenuation of hsp70 gene expression (Fargnoli et al., 1990; Pahlavani et al., 1995).

An aging-dependent increase of collagen-binding Hsp47 was displayed in the glomeruli, tubular epithelial cells, and interstitial cells in kidneys of old rats (Razzaque et al., 1998). In addition, Maiello et al., (1998) reported that the basal, unstimulated hsp70 mRNA in Wistar rat kidneys is increased in young (2-3 months) as 182% and old (22-27 months) as 167% compared with adults (6-11 months); it is likely that different mechanisms are responsible for the increased Hsp70 basal synthesis in both young and old animals. That is, differential chaperone induction in aged animals implies that it does not seem that a general mechanism is responsible for the impairment in chaperone transcription. Indeed, the level of HSF-1 as well as trimerization, phosphorylation, and nuclear translocation are usually unchanged during aging,

while binding of HSF-1 to the heat shock element (HSE) is decreased in aged hepatocytes as well as in myocardial cells (Locke and Tanguay, 1996; Heydari et al., 2000). On the contrary, HSF activation and HSE binding is preserved in rat skeletal muscle during aging (Locke, 2000). The exact mechanism of the defective activation or the tissue-specific differentiated chaperone induction is not known at this time, but it seems that a better adaptation capacity to various stresses during aging can greatly increase the chances to reach longevity.

1.3.4 DmHsp22, a mitochondrial sHsp with chaperone-like function

Recently, Morrow et al. have reported that over-expression of Hsp22, a small heat shock protein localized in mitochondria matrix, significantly extended lifespan approximately up to 32% in *Drosophila melanogaster* (Morrow et al., 2004a). On the contrary, the site-mutated deletion of *hsp22* gene in *Drosophila* resulted in approximately 40% decrease of mean lifespan (Morrow et al., 2004b). Another study reporting an increased mRNA level of *hsp22* in aged *Drosophila* suggests that expression of Hsp22 might be beneficial in preventing damages induced by aging and have an influence on the lifespan (King and Tower, 1999; Kurapati et al., 2000). The *in vitro* chaperone-like activity of Hsp22 compared to other small heat shock proteins in *Drosophila* is relatively high as shown in Fig. 1-8 (Morrow et al., 2006).

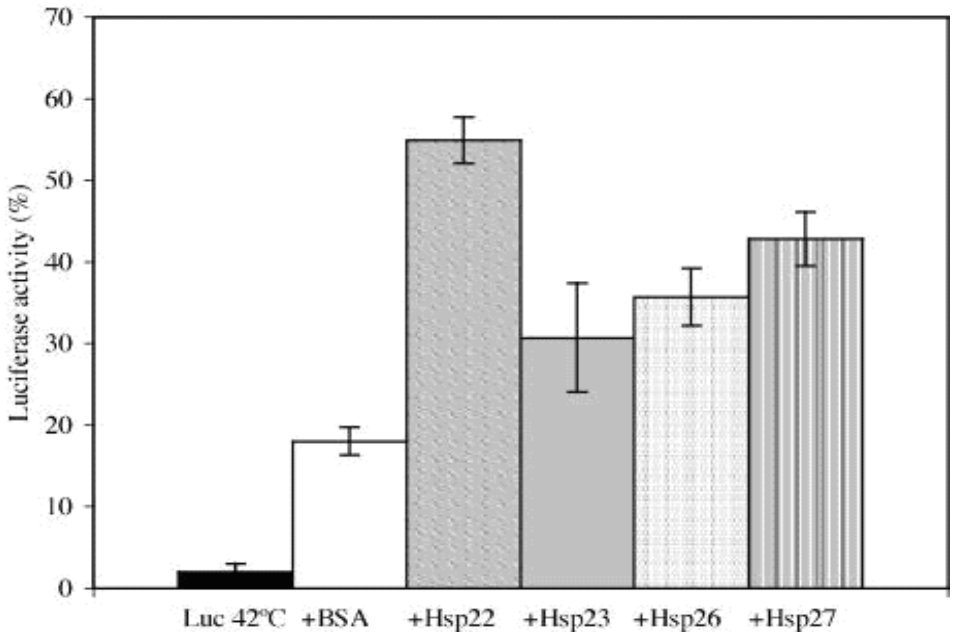
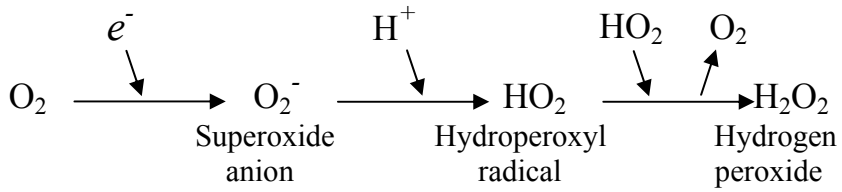


Fig. 1-8. *In vitro* chaperone-like activity of small heat shock proteins
(Reference: Morrow et al., 2006)

1.4 ROS and cellular homeostasis

1.4.1 ROS generation via mitochondrial respiratory chain and ROS scavenging enzymes

Under normal cellular condition, the major production of ROS is generated through the mitochondrial respiratory chain (Ambrosio et al., 1993). Between 0.4% and 4% of the oxygen consumed by mitochondria has been estimated to be converted into ROS (Giulivi et al., 1995; Gudz et al., 1997). In the mitochondrial matrix, oxygen plays a role as a final electron-acceptor transferring electrons from NADH, which is an electron-donor generated via TCA cycle by complex I-specific substrates such as glutamate or malate. In the case of NADH formed from pyruvate substrate via cytosolic glycolysis, they are transferred into mitochondrial matrix using glycerol 3-phosphate carrier or malate-aspartate shuttle, whereas, succinate donates electrons to complex II (Fig. 1-9). Electrons from complexes I and II are transferred to complex III by the common shuttle ubiquinone coenzyme Q (CoQ). The electrons reach oxygen, the terminal electron-acceptor, via cytochrome c and complex IV. This transfer of electrons leads to safe products (two molecules of H2O) during the respiratory chain, but partial reduction generates hazardous compounds. However, superoxide generated through free radical leakage is quickly converted to hydrogen peroxide that can diffuse to the cytoplasm and interact with redox active metals including iron or copper to further generate hydroxyl radical through Fenton or Haber-Weiss reactions (Lovell and Markesbery, 2007b). Protonation of superoxide anion yields hydroperoxyl radical (HO2), and these ROS form a cascade of damaging oxidative species, including the hydroxyl radical, hydrogen peroxide, peroxynitrite, maldonaldehyde, 4-hydroxynonenal, and so on (Ambrosio et al., 1993; Chance et al., 1979).



Ultimately, this electron flow generates an electrochemical gradient (both membrane potential and pH gradient). ATP synthetase (complex V) uses this electron-motive force to allow protons to re-enter into the matrix, then to drive phosphorylation of ADP to ATP (Walker, 1992). The substrates for phosphorylation, ADP and inorganic phosphate (Pi), enter the matrix via the ADP/ATP translocase (ANT) and the phosphate carrier. During the process of the respiratory chain, complex I and complex III of the electron-transport chain are major sites for ROS production (Sugioka, et al., 1988; Turrens and Boveris, 1980). In addition, Kudin et al. (2004) showed that the majority of ROS production into the mitochondrial matrix was from complex I. However, Starkov et al. (2004) have presented evidence that the main site of ROS release in the mitochondrial matrix may be the alpha-ketoglutarate dehydrogenase.

Natural defense mechanisms, including a manganese-dependent superoxide dismutase (MnSOD) exist within the mitochondrion to limit oxidative damage (Fridovich, 1995; Green et al., 2004). Additional forms of SOD exist in the cytoplasm and extracellularly to scavenge ROS outside the mitochondrion. Chen et al., (2003) reported that the released ROS from complex I (NADH dehydrogenase site) and complex III (Qi site) are directed toward matrix antioxidant defenses such as, manganese superoxide dismutase (MnSOD), glutathione (GSH), glutathione peroxidase (GPX), and glutathione reductase (GR). However, the Qo site of complex III releases the ROS toward the intermembrane space, away from matrix antioxidant defenses, favoring release from mitochondria (Fig. 1-9). Therefore, the ROS production and release, and the site of the free radical damage to the cell may differ depending on the location of mitochondrial inhibition (Sedensky and Morgan, 2006).

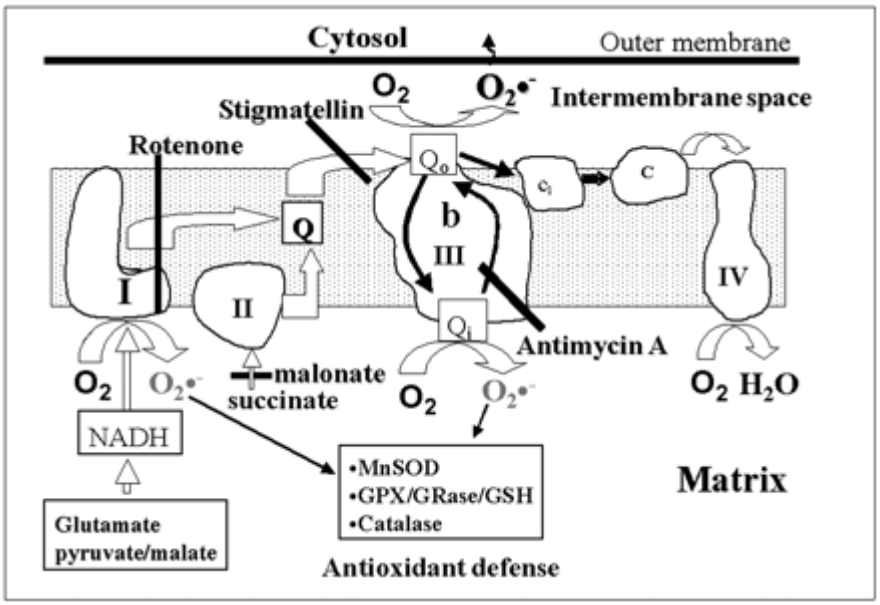


Fig. 1-9. Major sites of ROS generation, complex specific inhibitors and scavenging enzymes in the mitochondrial respiratory chain

(Reference: Chen et al., 2003)

1.4.2 Effect of ROS in signaling pathway

Some evidence implicates ROS as specific signaling molecules under both physiological and pathophysiological conditions and as essential factors to maintaining homeostasis in biological processes (Finkel and Holbrook, 2000). For example, ROS generation by phagocytic cells constitutes an essential host defense mechanism necessary to combat infection, and cytosolic ROS production in response to stimulation by growth factors is involved in regulating the proliferative response (Finkel, 1998). Moreover, under certain situations of metabolic stress, mitochondria-derived oxidants seem to function as signaling molecules in the biochemical pathways, which are involved in hyperglycaemia-induced abnormalities in glucose-induced activation of protein kinase C, formation of advanced glycation end-products, sorbitol accumulation and NFkappaB activation (Nishikawa et al., 2000). In addition, mitochondrial metabolism of pyruvate is demonstrated to activate the c-Jun N-terminal kinase (JNK) being triggered by released mitochondrial H2O2 (Nemoto et al., 2000). Although chronic ROS production may have deleterious effects, mitochondrial oxidants can also function acutely as signaling molecules to provide communication between the mitochondria and the cytosol by activating a novel metabolic regulatory pathway (Nemoto et al., 2000).

1.4.3 Oxidative damage to cellular compartments during aging and repair system

Regardless of how or where reactive free radicals are generated, an increase of intracellular oxidant levels during aging has been reported as factors not only triggering the activation of specific signaling pathways but also damaging to various cell components. Thus, these damages can influence on numerous cellular processes linked to aging and the development of age-related diseases (Finkel and Holbrook, 2000). The most vulnerable compartment is mitochondria because of their ROS generation combined with electron leakage through electron transport chain. There are several types of adductive materials presenting age-dependently increased levels, such as protein oxidations (Stadtman and Levine, 2000; Schöneich, 1999), advanced glycation end products (AGEs) (Kristal and Yu, 1992; Onorato et al., 1998), oxidized nucleic acids (Bohr et al., 1998), sterol oxidation products (Sevanian et al., 1991; Schröpfer, 2000), and lipid peroxidation (LPO) products.

1.4.3.1 Aging, protein oxidation and repair system

Protein oxidation results from the reaction with the reactive oxygen (nitrogen) species, which are produced by intracellular aerobic metabolism at the peroxisome and the mitochondria (Beckman and Ames, 1998), and are targeted at both amino acid side chains and peptidic backbone (Berlett and Stadtman, 1997). The oxidative damage to proteins can virtually affect all amino acids including sulfur-containing amino acids and aromatic amino acids (tyrosine, tryptophan or phenylalanine) being mainly susceptible to oxidation (Naskalski and Bartosz, 2000), and all of which can result in a wide variety of modified proteins and damaged products (Berlett and Stadtman, 1997).

Certain oxidation products of cysteine and methionine are reversible since they can be brought back to the reduced form of the amino acid within proteins by specific enzymatic systems which correspond to oxidized protein repair enzymes (Brot and Weissbach, 2000; Holmgren et al., 2005). However, these direct protein repair mechanisms for oxidized proteins are still very limited, as shown in cases listed at Table 1-3. The oxidation of methione residues in proteins results in the impairment of the protein function and loss of their activity, while their catalytical reversion by methionine sulfoxide enzyme allows a partial recovery of the protein function (Moskovitz, 2005). On the other hand, the major antioxidant enzymes involved in the protein repair systems, thioredoxin (Trx) and glutaredoxin (Grx) are small ubiquitous proteins

belonging to the thiol/disulfide oxidoreductase family, the members of which contain a redox active center made of two cysteines that can form a disulfide bridge upon oxidation. Oxidized thioredoxin is reduced by thioredoxine reductase (TrxR) such as selenocysteine and flavin containing enzyme in a NADPH dependent manner, while oxidized glutaredoxin is reduced by glutathione reductase (GR) also in a NADPH dependent manner. Ultimately, both Trx/TrxR and Grx/GSH/GR system are present in the cytosol and the mitochondria in eukaryotes (Friguet, 2006), and have been suggested to be involved not only in oxidized protein repair but also in cellular protection against oxidative stress as well as in redox signaling process.

Table 1-3. Direct protein repair mechanisms for oxidized proteins

Target site for repair	Repair mechanism
Thiol	Glutathione/thioltransferase system Thioredoxin/thioreductase system
Methionine sulfoxide	Methionine sulfoxide reductase Methionine reductase for free methionine
Cysteine sulfenic acid	Sulfiredoxin

(Reference; Poppek and Grune, 2006)

The most frequently hydroxylated or carbonylated amino acid derivatives however, are irreversibly oxidized by changing their secondary or tertiary structures and forming protein-protein covalent cross-link or non-covalent aggregates (Grune et al., 1997, Bader and Grune, 2006). During the process of covalent cross-linking of protein aggregates, non-protein components such as carbohydrates and oxidized lipids can react with protein adducts and thus increase the mass of oxidized aggregates (Berlett and Stadtman, 1997, Bader and Grune, 2006) (see Fig. 1-10). Ultimately, they are directed for degradation preferentially by the 20S proteasome in ATP- and ubiquitin-independent manner or by ubiquitin-26S proteasome pathway, because these protein aggregates are poor substrates for proteases, otherwise resulting in accumulation within cells (Davies, 2001; Bader and Grune, 2006).

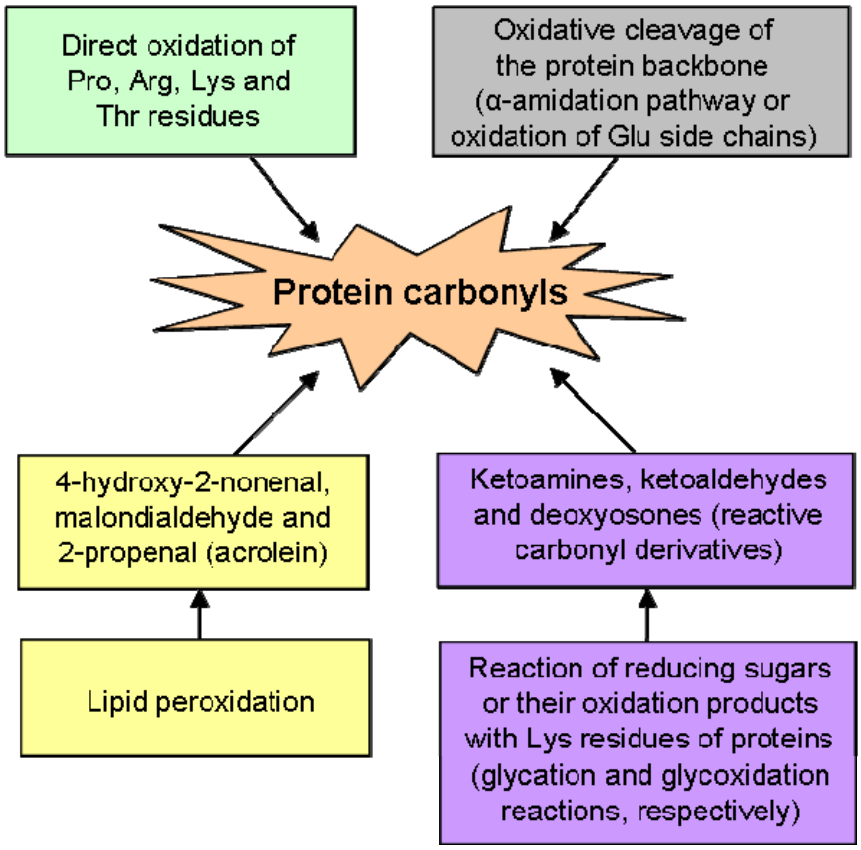


Fig. 1-10. Production of protein carbonyls

(References: Dalle-Donne et al., 2003)

A number of studies have shown increases in the intracellular concentrations of oxidized proteins as a function of age. Increases in protein carbonyls occur in rat hepatocytes, fruit fly, brain, and kidney of mice and in brain tissues of gerbils (Sohal et al., 1993; Sohal et al., 1995). In human, protein carbonyls increase with age specifically in brain, muscle, and human eye lens (Russell et al., 1987; Pansarasa et al., 1999; Mecocci et al., 1999; Smith et al., 1991). The rate of oxidation of proteins increases dramatically in the last third of lifespan and it can inactivate catalytic function as a physiological relevance (Starke et al., 1987; Starke-Reed and Oliver, 1989). In *Drosophila*, restricting flying increases lifespan, and this correlates with reduced protein carbonyls (Yan and Sohal, 2000). Interestingly, not all proteins are uniformly susceptible to oxidative damage. It was demonstrated that mitochondrial aconitase was particularly vulnerable to oxidative damage accompanying aging in *Drosophila* (Yan et al.,

1997). Similarly, mitochondrial adenine nucleotide translocase (Yan and Sohal., 1998), glutamine synthetase and creatine kinase (Smith et al., 1991; Oliver et al., 1990) were shown to be particularly vulnerable to protein oxidation. There is a large body of evidence implicating protein oxidation involvement in normal aging and neurodegenerative diseases (see Table 1-4). Oxidative damage is selective in attacking particular proteins preferentially and this can lead to inactivation of enzymatic activity or activation of kinase signaling pathways.

Table 1-4. Protein oxidation involved in age-related or neurodegenerative diseases

Disease	Oxidative modification	Reference
Alzheimer's disease	Protein carbonyls	Hensley et al., (1995)
	Oxidized glial glutamate transporter	Lauderback et al., (2001)
	4-hydroxynonenal protein modification	Ando et al., (1998)
	Protein 3-nitrotyrosine and dityrosine	Hensley et al., (1998)
	Nitration of MnSOD	Aoyama et al., (2000)
Parkinson's disease	Protein carbonyls	Alam et al., (1997)
	Oxidation of 6-hydroxy dopamine	Riobó et al., (2002)
	Nitration of MnSOD	Aoyama et al., (2000)
	3-nitrotyrosine	Good et al., (1998)
	Alpha-synuclein nitration	Giasson et al., (2000)
ALS	Protein carbonyls	Ferrante et al., (1997)
	hydroxynonenal modified protein	Pedersen et al., (1998)
	Nitration of MnSOD	Aoyama et al., (2000)
	3-nitrotyrosine	Tohgi et al., (1999)
Huntington's disease	3-nitrotyrosine	Tabrizi et al., (2000)

1.4.3.2 Aging, damage by lipid peroxidation

Lipid peroxidation is a complex process involving the interaction of reactive free radicals with polyunsaturated fatty acids, and finally results in a variety of very reactive electrophilic aldehydes that are capable of easily attaching covalently to proteins by forming adducts with cysteine, lysine, or histidine residues (Kopitz et al., 2004). Among the aldehydes formed, malondialdehyde (MDA) and 4-hydroxynonenal (HNE) represent the major products of lipid

peroxidation (Esterbauer et al., 1991), and several age-dependent degenerative diseases are reported to be accompanied by accumulation of intracellular granules (lipofuscin) composed by a complex of lipid peroxidation product-damaged protein (Brunk and Terman, 2002; Terman and Brunk, 2004).

For example, the increased lipid peroxidation in vulnerable regions of the mild cognitive impairment (MCI) brain and in the brains of patients with early Alzheimer's disease (EAD) (Lovell and Markesbery, 2007a) indicates that the brain may be particularly susceptible to free-radical-mediated lipid peroxidation caused by the presence of relatively high concentrations of redox active oxidants but relatively low antioxidant capacities. In addition, attack on polyunsaturated fatty acids by free radicals leads to structural damage to membranes and a generation of several aldehydic byproducts such as malondialdehyde (MDA) or α, ß-unsaturated aldehydes such as 4-hydroxy-2-nonenal (HNE) and acrolein. The HNE and MDA especially display high reactivity with nucleophiles including sulfhydryl groups of cysteine, histidine, and lysine (Esterbauer et al., 1991). There is a reported example on the damage to lysosomal membrane which can be induced by lipid peroxidation products (MDA, HNE). Thus, the inhibition of lysosomal hydrolytic activities causes a breakdown of the pH-gradient across the lysosomal membrane, causing severe cell damage and induction of apoptosis (Agha and Gad, 1995; Martinez, et al., 1990). Moreover, they are neurotoxic, can inhibit enzymes critical for neuron survival, and can induce changes in tau related to neurofibrillary tangles in AD (Gomez-Ramos et al., 2003; Liu et al., 2005).

1.5 Degradation of oxidative modified proteins

1.5.1 Lysosomal degradation

The lysosomal degradation pathway is mainly used for the removal of extracellular and autophagositized material. The cell takes up foreign protein materials, the targeted proteins for degradation are transported into the lysosomes, and are then exposed to cathepsin enzymes (Poppek and Grune, 2006). However, this lysosomal system has less selectivity once the protein adducts are inside the lysosomes, therefore the role of removal of oxidized proteins is less significantly considered (Kiffin et al., 2004). Nevertheless, it has been reported that activity of the lysosomal pathway is impacted by aging, and chaperone-mediated autophagy enhances translocation into lysosomes (Kiffin et al., 2004).

1.5.2 Proteolysis and proteasome system

Irreversibly damaged proteins are recognized by chaperones, and they are targeted for the site-specific cellular degradation such as by proteolysis in the mitochondria or by proteasome system in the cytosol. As a result of the aging process, the level and function of the proteasome/proteolysis system decrease and consequently the damaged proteins accumulate in the cells of aged organisms due to the degradation defects.

1.5.2.1 Mitochondrial protein degradation by proteolytic system

In the mitochondria, degradation of the oxidized protein adducts is mainly achieved by the ATP-stimulated Lon protease (Bota and Davies, 2002). Although it has been recently known that mammalian mitochondria contain three major ATP-dependent proteases; Lon, Clp-like and AAA proteases, the Lon protease is a better known, highly conserved protease found in prokaryotes and the mitochondrial compartment of eukaryotes (Bulteau et al., 2006). Additionally, the Lon protease is believed to play an important role in the degradation of oxidized mitochondrial matrix proteins such as aconitase, a TCA cycle enzyme (Bulteau et al., 2006; Bota and Davies, 2002; Bota et al., 2002). Down regulation of the human Lon protease results in disruption of mitochondrial structure, loss in function, and cell death (Bota et al., 2005). Moreover, Lon-deficient cells reveal aberrant mitochondrial morphology and the presence of electron dense inclusion bodies in the mitochondrial matrix (Bulteau et al., 2006). All imply the important role of the Lon proteolytic system for the degradation of oxidized

protein within the mitochondrial matrix and for the maintenance of mitochondrial structural and functional integrity.

1.5.2.2 Cytosolic and nuclear protein degradation by ubiquitin-proteasome system

Degradation of cytosolic and nuclear proteins is exerted by the cytosolic ubiquitin-proteasome system (Gauss et al., 2005). The signal that leads to degradation by this system is the covalent attachment of the conserved 76-residue polypeptide ubiquitin to the substrate proteins. Mono-ubiquitination serves as a signal for protein localization, modification, or interaction with partner proteins, and formation of poly-ubiquitin chains is necessary for subsequent degradation (Hershko and Ciechanover 1998; Pickart 2001). In the end, proteins that are poly-ubiquitinated by a Lys48-linked ubiquitin chain are targeted for degradation by the 26S proteasome (Gauss et al., 2005). Proteasomes consist of two subcomplexes: 20S core particle in a barrel-shaped complex that harbors threonine proteases with their active sites facing the central cavity (Gauss et al., 2005). The 19S caps flanking the entries of the 20S core particles mediate the recognition of poly-ubiquitinated degradation substrates and exhibit an ATP-dependent unfolding activity (Gauss et al., 2005). Therefore, the proteasome system represents a highly efficient and tightly regulated system of protein degradation through target protein recognition, transport into the central cavity, and digestion.

On the other hand, age-related impairment of the proteasome function has been evidenced in a wide range of organs or cell types (Friguet et al., 2000). In addition, transcriptome analysis of both human dermal fibroblasts and mouse skeletal muscle has indicated an age-related decreased expression of several 20S and 26S proteasome subunits (Lee et al., 1999a; Ly et al., 2000). Interestingly, Bulteau et al. (2002) reported that strongly inhibited proteasome activity in the hearts of old rats has partly been recovered upon purification of proteasome, suggesting the existence of endogenous inhibitors in the homogenates that are removed during proteasome purification. Therefore, the decline of proteasome activity with age seems to be dependent on several inhibition mechanisms: inactivation of proteasome through direct subunit modification, or decreased expression of certain proteasomal subunits, or increased endogenous inhibitors such as cross-linked protein adducts (Friguet, 2006).

1.6 Objectives

Aging is a complex process regulated by multiple pathways, and resulting in a decreased capacity of cells to tolerate and respond to various forms of stresses leading to damages such as protein aggregation in various components of the cell. Among many proteins influencing lifespan, most heat shock proteins (Hsps) have been known to increase stress resistance but also chaperone function to support protein folding. Thus, Hsps as chaperone are likely important players in the aging process by preventing protein denaturation and aggregation. Moreover, in aging studies, the fruit fly *Drosophila melanogaster*, is a very useful species because of its relatively short life cycle, easy handling, and fully supported genome-wide and proteome database.

Recently it has been reported that over-expressing a small mitochondrial heat shock protein, Hsp22, is significantly beneficial to prolong the lifespan of *Drosophila melanogaster* (Morrow et al., 2004a & 2004b). This protein is localized in the mitochondrial matrix and has a function of chaperone-like activity in vitro (Morrow et al., 2006). Up to now, the detailed mechanism by which Hsp22 exerts its effect on longevity and whether this mechanism is linked to already known aging mechanisms or not, is unclear.

To address these questions, first we examined differences in physiological functions between Hsp22 over-expressing flies and Hsp22 normal-expressing control flies, which could be induced by Hsp22 over-expression. Mainly, age-dependent protein profile, protein carbonylation, and stress resistance (paraquat oxidant / starvation) were compared.

Secondly, we compared the relative abundance of the mitochondrial proteome between Hsp22 over-expressing flies and control flies. As a proteome analysis on whole cell extracts tends to miss relatively low-abundance proteins and their possible significance, compartmentalization of the eukaryotic cell can reduce the complexity of protein composition and yield higher specificity than that of whole cells. In addition, alterations in mitochondrial proteome are expected to reflect main targets which are associated with aging or anti-aging process. Mitochondrial localization of the Hsp22 protein has a higher potential to influence the mitochondrial proteome. Thus, we isolated mitochondria in adult flies and confirmed their

purity by identifying their protein composition using LC-MS/MS. At the same time, activities of mitochondrial respiratory enzymes, in Hsp22 over-expressing flies and in control flies can give insights on energy production and its consequent effects during aging process.

Thirdly, we performed a genome-wide transcriptional analysis by comparing Hsp22 over-expressing long-lived flies with normal-lived control flies. Understanding the differential profile of transcriptome in the long-lived flies having Hsp22 over-expression may underline key mechanisms involved in lifespan extension and furthermore strategies to counter the aging process.

Chapter 2. *Drosophila melanogaster* over-expressing Hsp22 have a prolonged lifespan and a greater stress resistance

2.1 Abstract

Heat shock proteins (Hsps) generally have involvement into stress response and protection from molecular damage. Many Hsps play a role of molecular chaperone that functions in protein folding or refolding and/or facilitate protein translocation across cellular membranes. The Hsps proteins have tissue-specific or developmental stage-dependent variable expressions during the normal aging process and their over-expression is often associated with a prolonged lifespan in many organisms.

One of the small heat shock proteins, Hsp22 in *Drosophila melanogaster* has demonstrated a beneficial effect on longevity, showed a chaperone-like activity *in vitro*, and is specifically localized in mitochondrial matrix. Based on the free radical theory of aging (Harman, 1956), the aging process is closely related to deleterious effect by ROS production in mitochondria. Therefore, it is hypothesized that evaluating the differences in lifespan, stress responses and protein oxidation, caused by Hsp22 over-expression might give insight on the possible anti-aging mechanism by this mitochondrial chaperone protein.

The ubiquitous over-expression of endogenous Hsp22 was performed using GAL4/UAS system, and flies over-expressing Hsp22 showed ≈32% of increased mean lifespan, better resistances to starvation and oxidative stress than control flies, and decreased level of protein oxidation during aging.

2.2 Introduction

The induction of heat shock proteins (Hsps) can be associated with their functions in stress tolerance and protection from molecular damage. According to their molecular weight and sequence homology, the Hsps are classified into several families. Among them members of Hsp70, Hsp90 and Hsp60 families play a prominent role as molecular chaperone being involved in protein folding or refolding and/or protein translocation across cellular membranes. This chaperone activity is ATP-dependent and often involves co-chaperones. The small heat shock proteins (shsps) are a more diverse group with molecular weights ranging from 15 ~ 45kDa. They have a conserved α-crystallin domain and have been found in different subcellular localizations (Haslbeck and Buchner, 2002; Michaud et al., 2002; Van Montfort et al., 2001b), and several shsps have been shown to have a chaperone-like activity *in vitro*.

According to Harman's free radical theory of aging (Harman, 1956), the aging process is associated with the accumulation of deleteriously damaged proteins resulting in conformational alteration and/or enzymatic inactivity (Finkel and Holbrook, 2000; Gershon and Gershon, 1970; Stadtman, 1992). Various Hsps proteins have tissue-specific or developmental stage-dependent variable expression during the normal aging process (Fleming et al., 1988; Marin et al., 1993; Wheeler et al., 1995; Morrow and Tanguay, 2003; Singh et al., 2007) and their over-expression has been often associated with a prolonged lifespan in many organisms (Tatar et al., 1997; Kurapati et al., 2000; Morrow et al., 2004a). In addition, their dysfunction in protein folding has been related to neurological diseases due to the limited proliferation of neurons, whereas increased chaperone induction often delays the onsets or diminishes the symptoms of such age-associated neuronal degenerative diseases (reviewed in Sőti and Csermely, 2002b; Morrow and Tanguay, 2006).

Genetic selection for longevity in *Drosophila* uncovered increased expression of hsp22 in young adults (Kurapati et al., 2000). The over-expression of Hsp22 had led to an extended lifespan and increased resistance to oxidative stress (Morrow et al., 2004a), while the absence of Hsp22 expression resulted in decreased lifespan (Morrow et al., 2004b). Hsp22 is localized in the mitochondrial matrix (Morrow et al., 2000) and has a chaperone-like activity *in vitro*

(Morrow et al., 2006). The detailed mechanism by which Hsp22 influences longevity remains unknown.

In the present study, the ubiquitous over-expression of Hsp22 in *Drosophila melanogaster* was demonstrated using the GAL4/UAS system. Then, the differential stress responses and pattern of protein damage were compared between Hsp22 over-expressing and control flies during aging in order to understand the function of Hsp22 during aging.

2.3 Method

2.3.1 *Drosophila* strains and Hsp22 over-expression by GAL4/UAS system

Since Fischer et al. (1988) demonstrated that GAL4 expression was capable of stimulating transcription of a receptor gene under UAS (Upstream Activating Sequences) control in *Drosophila melanogaster*, the GAL4/UAS system has been applied as an advantageous tool for targeted gene expression. This system was applied for ubiquitous over-expression of endogenous Hsp22 by using *actin-GAL4* and *UAS-EP(3)3247* lines in present study. The *actin-GAL4* line (Flybase ID: FBti0012293) contains 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* line (Flybase ID: FBti0011419, Rørth, 1996) contains a UAS-including P-element that is inserted 643bp upstream of the *hsp22* translation initiation codon (FlyBase Genome Annotators, 2002-2003) on the third chromosome. Activation of the UAS by GAL4 results in Hsp22 over-expression as schematically shown in Fig.2-1. Siblings which were obtained from the crossing between *actin-GAL4/cyo* male flies and *EP(3)3247/TM3sb* female flies were used in subsequent experiments. For a wild type fly, male *W1118* was used.

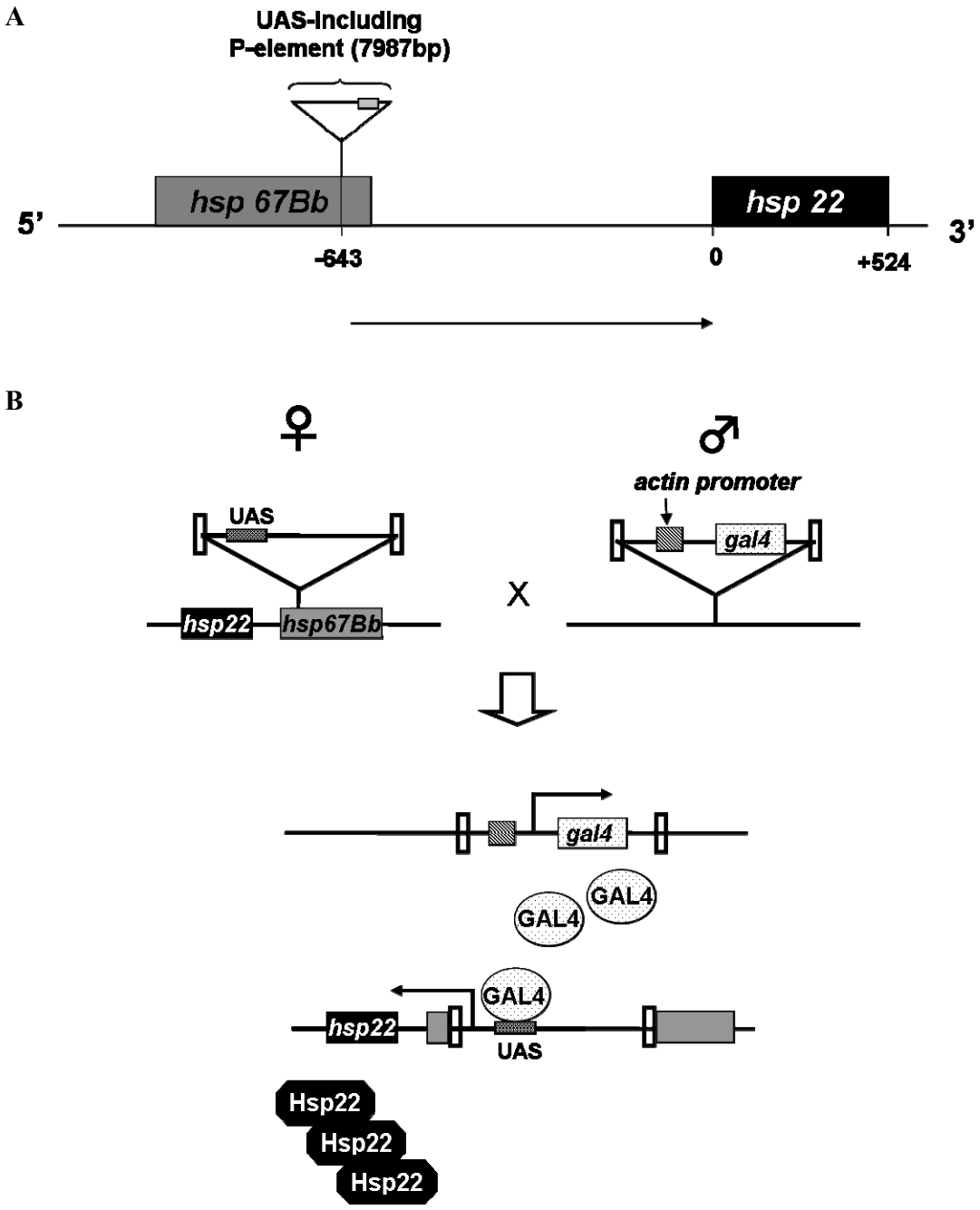


Fig. 2-1. Gene map of EP(3)3247 line and schematic design for Hsp22 over-expression by GAL4/UAS system

A) EP(3)3247 strain contains a UAS-including P-element insertion at 643bp upstream of the *hsp22* translation initiation codon. B) By crossing females of EP(3)3247 strain and males of a*ctin*-Gal4 strain, activation of UAS by *actin*-promoted GAL4 protein drives Hsp22 induction.

2.3.2 Fly incubation and longevity test

Among the siblings, 500 male flies for each of *actin-GAL4;EP(3)3247* (*Hsp22+;* Hsp22 over-expressing flies) and *actin-GAL4;TM3sb* (*control;* Hsp22 normal-expressing flies) were collected within 24h after eclosion in jars and transferred into tubes (20 flies per tube). 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, 1.8% (v/v) tegosept. Every 3-4 days, the survival rate was scored for longevity test, and dead flies were removed by replacing with new tubes containing a fresh medium.

2.3.3 SDS gel electrophoresis and immunoblotting

Fly samples were managed under the same conditions in longevity experiment. Sample collection was done at same chronological ages for the male flies of *Hsp22+* and *control* flies. The collected flies were immediately frozen and kept at -80°C until use. Protein extracts from flies were separated on 12% SDS-PAGE as described in Marin et al. (1996). Briefly, five flies were homogenized directly in 250µl of SDS lysis buffer (0.075 Tris-HCl (pH 6.8), 2.3% (w/v) SDS, 5% (v/v) bromophenol blue), and heated at 95°C for 5min. The SDS polyacrylamide gels were prepared as outlined by Thomas and Kornberg (1975), with modification in the pH of the running buffer (8.5 instead of 8.8) and in the acrylamide:bis ratio (30:0.8 instead of 30:0.15). These conditions resulted in a better resolution for the sHsps. Following transfer on nitrocellulose membranes, a Western blot was performed using polyclonal rabbit antibodies such as; Hsp22 (#36, 1/2500; Morrow et al., 2000), Hsp60 (#37, 1/5000), Hsp70 (#799, 1/5000; Tanguay et al., 1993) and DmHSF (943bb, 1/5000; Westwood et al, 1991). Monoclonal antibodies against *Drosophila* sHsps were applied for Hsp23 (7B12, 1/5000; Marin et al., 1993), Hsp26 (10D3, 1/100; Marin et al., 1993), Hsp27 (2C8, 1/100; Marin et al., 1993), and Hsp83 (3E6, 1/100; Carbajal et al., 1990). Peroxidase-conjugated secondary antibodies of goat anti-rabbit or goat anti-mouse (1/10,000, Jackson ImmunoResearch laboratories, West Grove, PA) were used. Chemiluminescent detection was done using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instruction.

2.3.4 Hsp22 over-expression by different GAL4 promoters

To compare the pattern of Hsp22 over-expression under various promoters, the activation of GAL4/UAS system was examined for three different GAL4 lines of *actin-GAL4* (ubiquitous), *tub-GAL4* (ubiquitous) and *da-GAL4* (ubiquitous). The GAL4-containing male flies were crossed with female flies of UAS-containing *EP(3)3247* line. From the progeny, males of *actin-GAL4*;*EP(3)3247*, *tub-GAL4;EP(3)3247* and *da-GAL4;EP(3)3247* were collected at 0-12hrs, 12-24hrs, 24-36hrs, 36-48hrs, day 5 and day 10. Flies were maintained as described in section 2.3.2, and proteins were separated on 12% SDS-PAGE. Temporal patterns of Hsp22, Hsp70 and Hsp83 were detected following immunoblotting procedure.

2.3.5 Protein carbonylation assay

Highly reactive free radical species can oxidize proteins, and the most general indicator and by far the most commonly used marker of protein oxidation is protein carbonyl content (Stadtman and Oliver, 1991). Protein carbonylation was detected by using the chemical and immunological reagents of the OxyBlot^{TM} Oxidized Protein Detection Kit (Intergen, USA). Flies for protein samples were homogenized in a buffer (210mM mannitol, 70mM sucrose, 1mM EGTA, 5mM HEPES, pH 7.2) containing 2% mercaptoethanol to protect from further carbonylation during preparation. The carbonyl groups in the protein side chains are derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH) by following the manufacturer's instruction. The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis, and detected through the western blot procedure using anti-DNP antibodies.

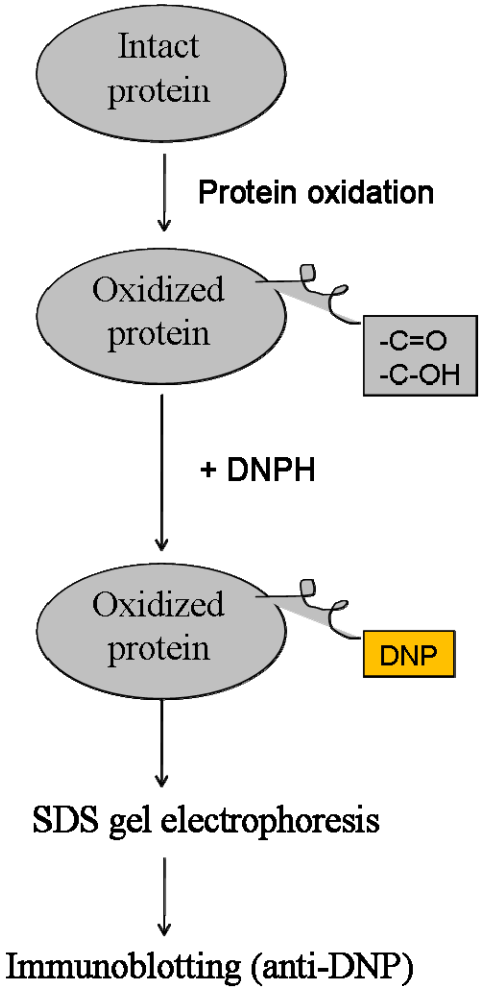


Fig. 2-2. Schematic overview of protein carbonylation assay

Oxidized protein forms carbonyl groups in amino acid side chain which is more vulnerable to reactive free radicals. The protein carbonylation assay utilizes the reaction between 2,4-dinitrophenylhydrazine (DNPH) and protein carbonyls. DNPH reacts with protein carbonyls, and produces 2,4-dinitrophenylhydrazone (DNP) which can be detected through the western blot procedure using anti-DNP antibodies.

2.3.6 Analysis of starvation resistance

Approximately 100 flies were used in this experiment. 5-day-old flies were placed into empty clean vials (10 flies per vial) at 25°C, and time-dependent survival rate was calculated.

2.3.7 Analysis of paraquat resistance

The experimental procedure for paraquat (methyl viologen, Sigma, Ontario, Canada) feeding was modified as described by Humphreys et al. (1996). In advance, whatman 3M filter strips (10 × 40 mm) were placed in clean empty vials and paraquat was solubilized to a proper stock concentration avoiding light exposure. All reagents were freshly prepared. For a control feeding, 200μl of 1% sucrose was absorbed in the filter strips. The reagents for paraquat feeding were prepared by mixing 1 volume of 2% sucrose stock and 1 volume of paraquat stock (2×) in a dark room, and were soaked to the filter strips as in control feeding. The flies used for age-dependent response were placed in empty vials (10 flies per vial) for 6h before this test, and then exposed to paraquat feeding. The range of paraquat concentration and exposure time was adjusted according to the age-dependent sensitivity. For instance, 1-day-old flies were exposed to paraquat ranging 0 ~ 40mM for 72h (with refreshing filter strips every 24h), 7-day-old flies to 0 ~ 40mM for 24h, 25-day-old flies to 0 ~ 20mM for 24h, and 60-day-old flies to 0 ~ 4mM for 24h. Flies were kept in the dark during paraquat treatment, and the survival rate was scored and surviving flies collected for protein analysis. Approximately 100 flies were used in single test at each concentration and this experiment was repeated three times.

2.4 Results

2.4.1 Hsp22 over-expressing flies display a ≈32% extension of mean lifespan

To understand the effect of Hsp22 over-expression on aging in *Drosophila melanogaster*, the flies having Hsp22 induction by GAL4/UAS system and the corresponding control flies were examined for lifespan. As shown in Fig. 2-3, the *Hsp22+* flies showed significantly extended lifespan compared to the *control* flies. The mean lifespan at 50% survival was day 60 and day 79, respectively for *control* and *Hsp22+* flies. This indicates an approximate 32% mean lifespan extension in *Hsp22+* flies.

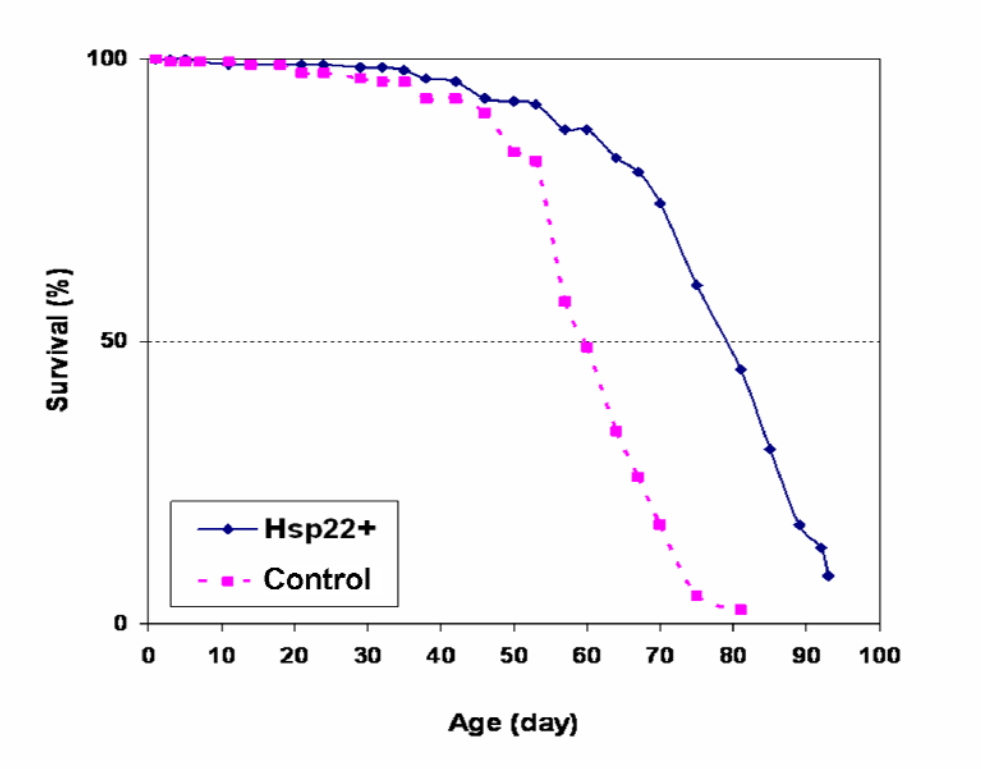


Fig. 2-3. Prolonged lifespan in Hsp22 over-expressing flies

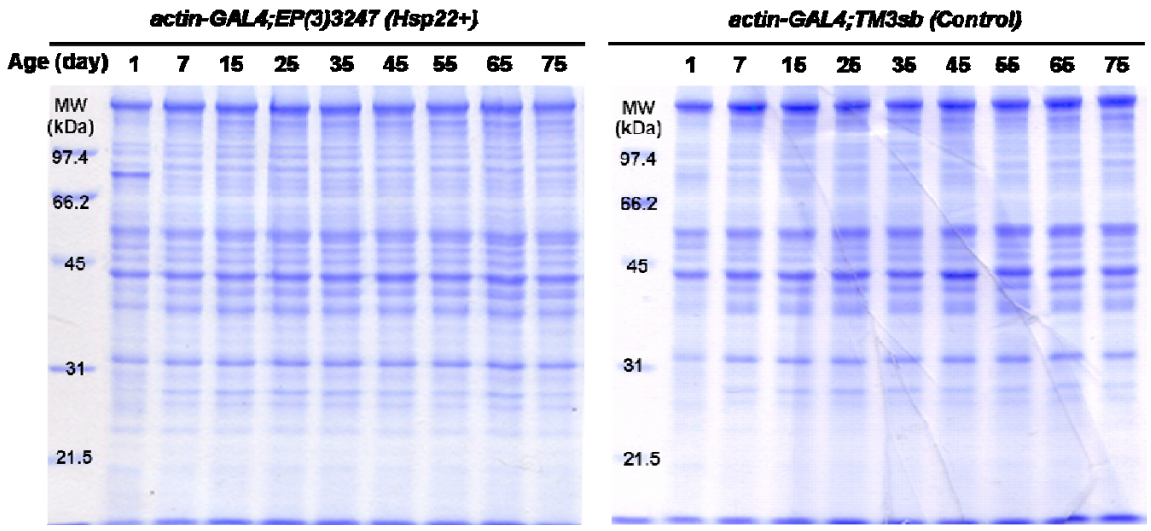
Hsp22+ indicates male of *actin-GAL4;EP(3)3247* and *control* indicates male of *actin-GAL4;TM3sb*. The flies were maintained with normal food at 25°C, and the survival rate was scored every 3-4 days.

2.4.2 Different pattern of heat shock proteins in Hsp22 over-expressing and control flies during aging

The *Hsp22+* and *control* flies were sampled during the aging process, and equivalent proteins of fly homogenates were analyzed for the expression of various Hsps including Hsp22. The age-dependent protein profile and western blotting image on Hsps are presented in Fig. 2-4. Interestingly, Hsp22 was not constantly expressed during aging and it was rather variable depending on the age. More specifically a different expression of Hsp22 between *Hsp22+* and *control* flies was found at the early age of adult fly (compare day 1 and 7 of Hsp22+ and control). The *Hsp22+* flies had a large amount of Hsp22 at the beginning of lifespan, whereas the *control* flies did not show the expression of Hsp22. The amount of expressed Hsp22, however, was remarkably increased from mid age (around age of day 45) in both *Hsp22+* and *control* flies although they had a different pattern of expression.

On the other hand, Hsp23 was expressed just at the beginning of adult life in *Hsp22+* flies, as well as in *control* flies. Both flies also displayed similar abundance of Hsp26 and Hsp27 at the beginning of lifespan, but the later pattern of these proteins was very different between *Hsp22+* and *control* flies depending on the age (Fig. 2-4). Relatively, *Hsp22+* flies constantly expressed Hsp26 and Hsp27 during aging whereas the control flies showed a significant decline of both of these sHsps. With regards to Hsp60, Hsp70 and HSF, the differences between *Hsp22+* and *control* flies in the pattern of expression were rather small although both of the flies showed slight alteration in protein abundance depending on aging procedure. In the case of Hsp83, the *Hsp22+* and *control* flies showed different pattern. The *Hsp22+* flies had an almost constant expression of Hsp83 while the *control* flies showed very rapid decrease in expression of Hsp83 during aging.

A



B

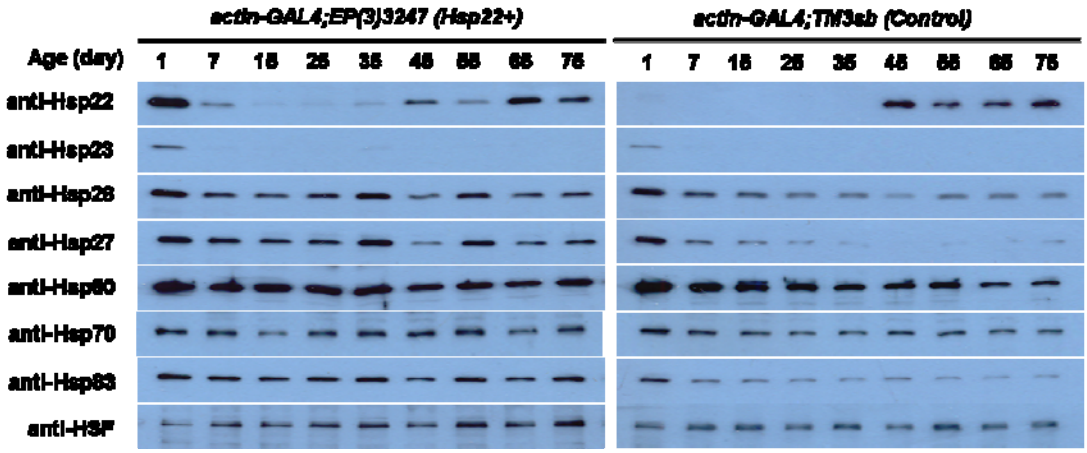


Fig. 2-4. Protein profile and pattern of heat shock proteins expression during aging in Hsp22 over-expressing flies and Hsp22 normal-expressing flies

A) coomassie blue stained gels, B) proteins were transferred to nitrocellulose membranes and blotted with antibodies against the different Hsps

2.4.3 The pattern of Hsp22 over-expression induced by different GAL4 promoters is similar at early adulthood

Unexpectedly, the pattern of Hsp22 induction by GAL4/UAS under the presence of ubiquitous *actin* promoter in flies appeared not to be constant and seemed to be more intensive at the early lifespan of adult flies as shown in Fig. 2-4. Therefore, the patterns of Hsp22 induction using three different GAL4 promoters were compared to understand whether this phenomenon was promoter specific or general. Two other ubiquitous promoters, the one of the tubulin genes (*tub*) and daughterless (*da*) were tested. The results showed that the overall patterns of Hsp22 induction by these different promoters were similar although there were differences in their abundance. The time-dependent expression of Hsp22 indicated very early induction within 48 hrs and then a decrease was observed at day 5 and day 10 (Fig. 2-5). On the other hand, the patterns of Hsp70 and Hsp83 were not so different among the flies containing *actin-, tub-* and *da-* promoter, although the relative abundances of Hsp70 and Hsp83 were different among the flies. These proteins however, did not have a rapid decline as shown in the case of Hsp22.

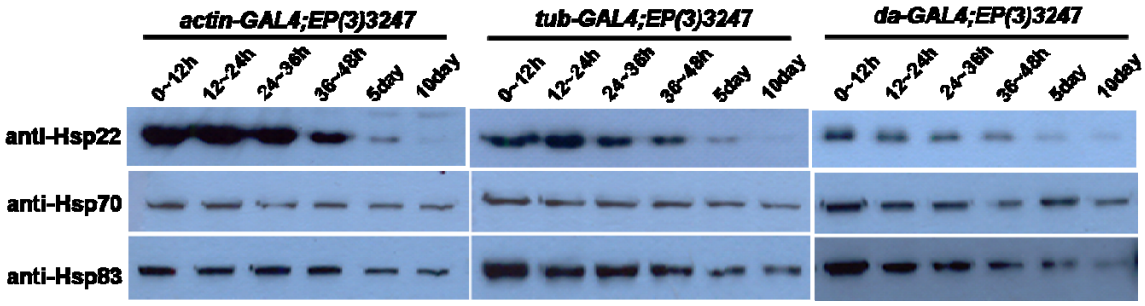


Fig. 2-5. Similar pattern of Hsp22 over-expression induced by different GAL4 promoters
Different sets of the activation of GAL4/UAS system were demonstrated using three different GAL4 lines of actin-GAL4 (ubiquitous), tub-GAL4 (ubiquitous) and da-GAL4 (ubiquitous). The male flies having different GAL4 promoter drivers were crossed with females of UAS-containing EP(3)3247 line. From the progeny, males of actin-GAL4;EP(3)3247, tub-GAL4;EP(3)3247 and da-GAL4;EP(3)3247 were applied to compare time-dependent expression of Hsp22. The patterns of Hsp70 and Hsp83 were used as internal control for Hsp22 and the external control among different flies.

2.4.4 The Hsp22 over-expressing flies have a better ability to regulate damaged proteins as shown by a decline of proteins carbonylated during aging

To examine the degree of oxidative modification of proteins in *Hsp22+* and *control* flies during aging, protein carbonylation assays were performed. The pattern of carbonylated protein displayed a broad spectrum of molecular weight (Fig. 2-6). There was protein-size specific variation in the abundance of carbonylated proteins depending on age and flies. However, the most significant carbonylation in protein was detected at the early stage of lifespan in *Hsp22+* flies. Notably, the overall abundance of carbonylated protein in 1-day-old *Hsp22+* flies was higher than that of 1-day-old *control* flies. Higher degree of protein carbonylation was also shown in 25-day-old *Hsp22+* flies relative to 25-day-old *control* flies. Interestingly, the protein carbonylation in *Hsp22+* flies however, was gradually reduced in an aging-dependent way whereas, the *control* flies displayed an almost constant abundance in main protein carbonylation, or slightly increased abundance in overall protein carbonylation during aging. Therefore, the regulation of carbonylated proteins in *Hsp22+* flies seems to be more effective than that of carbonylated proteins in *control* flies during aging process.

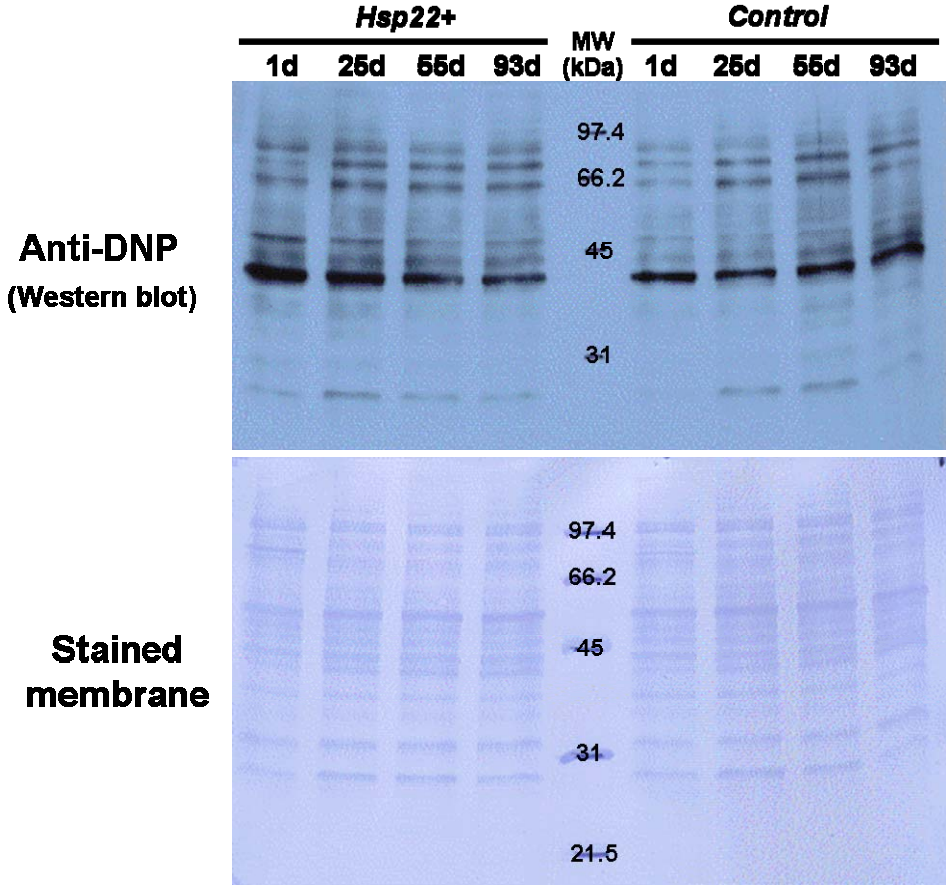


Fig. 2-6. Protein carbonylation detected in Hsp22+ and control flies during aging

Carbonyl groups in protein side chains, that are indicating oxidative protein modification were derivatized to 2,4-dinitrophenylhydrazone (DNP) by reaction with 2,4-dinitrophenylhydrazine (DNPH). The DNP-derivatized protein samples were separated by polyacrylamide gel electrophoresis, and the carbonylated proteins were detected by Western blot using anti-DNP antibodies.

2.4.5 Hsp22 over-expressing flies have an increased resistance to starvation stress

To examine the influence of Hsp22 over-expression under stress condition, the over-expressing and normal-expressing Hsp22 were compared for their resistance to starvation stress. As Hsp22 over-expression seemed to be more significant at the early lifespan in the *Hsp22+* flies, 5-day-old flies were used in this experiment. As shown in Fig. 2-7, the exposure time to result in 50% survival rate in *Hsp22+* flies was approximately doubled compared to that of the corresponding controls. Furthermore, the maximal total survival time under starvation was 65±7h in *Hsp22+* flies, while the control and wild type flies were able to survive until 42±7h.

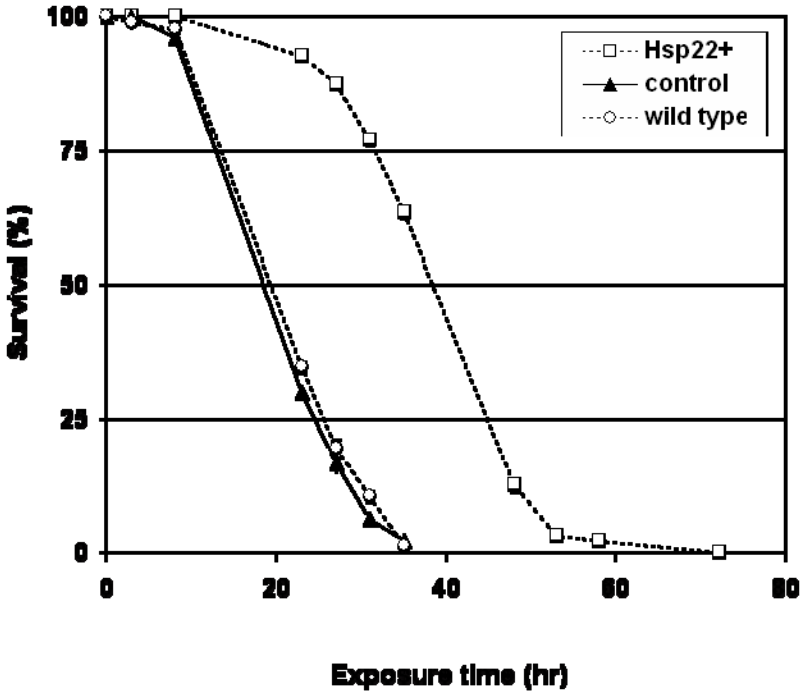


Fig. 2-7. Hsp22 over-expressing flies have increased resistance to starvation stress

5-day-old flies with Hsp22 over-expression (*Hsp22+*) and without Hsp22 over-expression (*control* and *W1118*) were exposed to starvation at 25°C.

2.4.6 Hsp22 over-expressing flies have an increased resistance to oxidative stress

During aging, organisms are exposed to oxidative stresses that are produced in intracellular metabolic processes and cause deleterious effect. Therefore, the lifespan extension in Hsp22 over-expressing flies may indicate a beneficial function of Hsp22 over-expression against oxidative stress. Age-dependent resistance to paraquat-inducing oxidative stress was investigated in *Hsp22+* and *control* flies. The flies at variable ages were treated with different concentrations of paraquat. Young flies were less sensitive to paraquat concentration by showing higher level of lethal concentration compared to old adult flies. Flies for the test were starved for 6h before paraquat treatment. The 6h-starvation did not influence the flies' survival rate as shown in the starvation experiment of Fig. 2-7.

Overall, *Hsp22+* flies had better paraquat-resistance than *control* flies (Fig. 2-8). The difference in survival between *Hsp22+* and *control* flies was larger at day 7 and day 25 than at day 1 and 60. The deleterious effect at paraquat under 1-day-old condition in both *Hsp22+* and *control* flies was increased corresponding to prolonged exposure-time. Thus, the exposure time was adjusted according to the age-dependent sensitivity, as 72h in the case of 1-day-old flies and 24h in the other cases. The lethal dose for 50% survival (LD_{50}) of paraquat in the *Hsp22+* flies was higher than that in the *control* flies (Table 2-1). Both flies also displayed age-dependent decline of $LD_{50;}$ however, the *Hsp22+* flies showed relatively very small decrease of LD_{50} value at the early ages of day 1 and 7.

Table 2-1. Aging dependent LD_{50} of paraquat in Hsp22+ and control flies

Age	LD_{50} of paraquat (mM)	
	Hsp22+ flies	Control flies
Day 1	27.6	23.0
Day 7	25.8	14.0
Day 25	12.3	7.5
Day 60	2.0	1.7

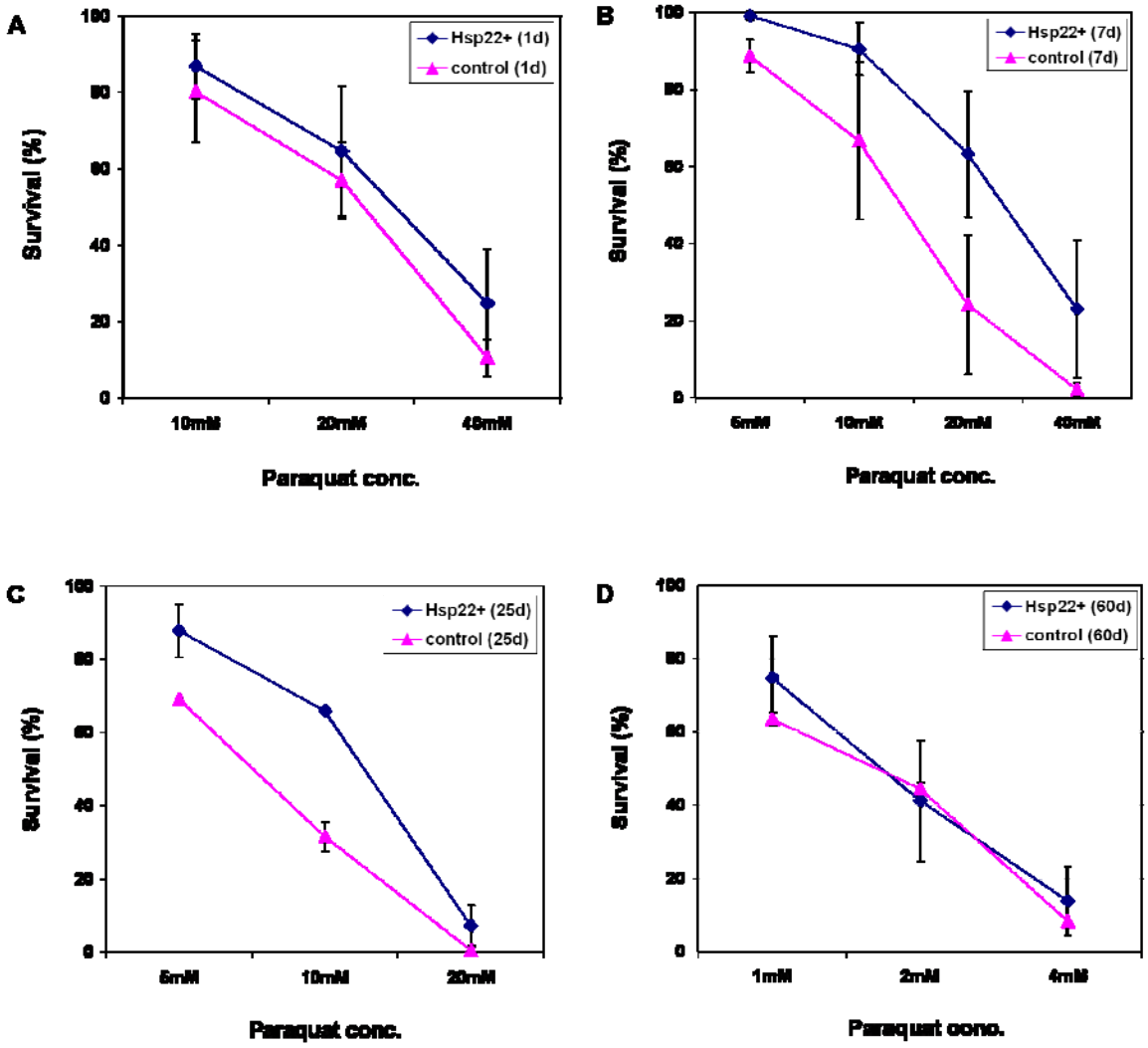


Fig. 2-8. Age-dependent paraquat resistance in Hsp22+ and control flies

1-day-old flies (A), 7-day-old flies (B), 25-day-old flies (C), and 60-day-old flies (D) were starved for 6h before being exposed to paraquat (72h for 1-day-old flies and 24h for the others) at the indicated concentrations.

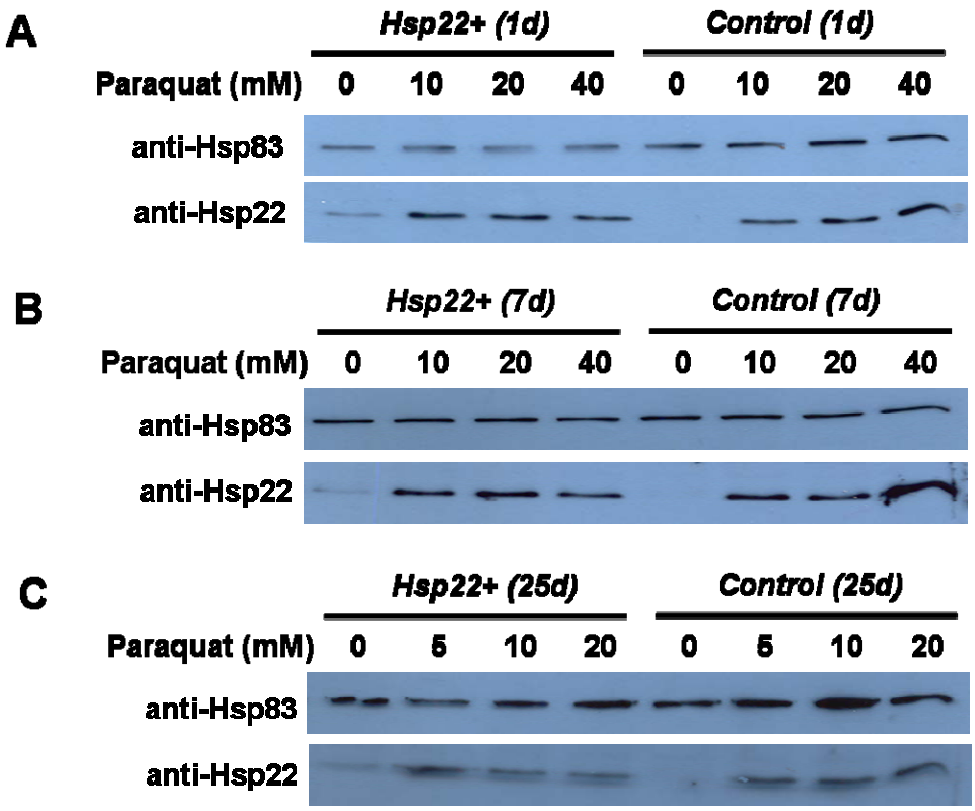


Fig. 2-9. Hsp22 induction by response to paraquat in Hsp22+ and control flies

1-day-old flies (72h exposure time) in A, 7-day-old flies (24h) in B, and 25-day-old flies (24h) in C were previously starved for 6h, exposed to paraquat for indicated exposure time. In this experiment, flies exposed to 0mM paraquat were previously starved, and treated as same procedure with flies under other concentrations. The flies surviving from treatment were used to analyze protein expression.

Western blot analysis was performed using flies surviving from paraquat treatment. As shown in Fig. 2-9, the *Hsp22+* flies had increased Hsp22 expression under paraquat exposure. The amount of expressed Hsp22, however, was not significantly influenced by paraquat concentration. The *control* flies showed no Hsp22 expression in the absence of paraquat, but they displayed Hsp22 induction as abundant as that of *Hsp22+* flies at the paraquat concentrations of 5 ~ 40mM. Both *Hsp22+* and *control* flies had a quite constant expression of Hsp83, regardless of paraquat treatment. Therefore, Hsp22 is a protein very responsive to oxidative stress, but not significantly changed according to the concentration of paraquat.

2.5 Discussion

Flies over-expressing Hsp22 using the GAL4/UAS system showed approximately 32% increase of mean lifespan compared to the control flies. The pattern of Hsp22 induction was not influenced by different type of ubiquitous promoters for GAL4 drivers. The abundance of Hsp22 expression in Hsp22 over-expressing flies declined at the early lifespan and again increased during mid lifespan. On the other hand, control flies expressed Hsp22 at the later stage of lifespan but not at the early phase of lifespan. The Hsp22 over-expressing flies also showed consistent differences in the expression of other Hsps such as Hsp26, Hsp27 and Hsp83.

The Hsp22 over-expressing flies had a better ability to survive under the starvation condition suggesting that the Hsp22 over-expressing flies may have certain advantages in regulating their metabolism. In the case of oxidative stress caused by paraquat, the Hsp22 over-expressing flies showed better resistance than the control flies. Especially, at the earlier ages (day 1 and day 7), the inhibitory effect caused by paraquat in the Hsp22 over-expressing flies was less severe than in control flies. For instance, the LD_{50} of paraquat in Hsp22 over-expressing flies was 27.6mM for 1-day-old and 25.8mM for 7-day-old. Thus the control flies had only 50% of surviving populations by exposure to 23.0mM paraquat for 1-day-old and 14.0mM paraquat for 7-day-old. In both Hsp22 over-expressing and the control flies, the Hsp22 protein was very sensitively induced by oxidative stress regardless of the paraquat concentration while Hsp83 did not appear to be significantly changed. In addition to the resistance against the oxidative stress, the Hsp22 over-expressing flies actually demonstrated a better capacity to regulate the oxidized proteins by showing a decrease of accumulation of damaged proteins during aging.

Since Harman proposed that aging could be attributed to deleterious effects of free radicals on various cell components (Harman, 1956), the major involvement of mitochondria in aging has been consistently hypothesized. A close relationship between the oxidative damage caused by ROS and the metabolic rate based on mitochondrial respiratory enzymes seems to exist. Perhaps, the induction of Hsp22 during aging may be a response to oxidative stress. Therefore, the early over-expression of Hsp22, demonstrated by GAL4/UAS system in the long-lived flies may contribute to the different level of protein damage from the beginning of lifespan. Although the exact mechanism of the age-associated defective activation caused by ROS are

still unclear, a reasonable hypothesis would be that a better capacity of regulating cellular modification under cell stress conditions during aging can increase the chance of longevity.

The expression of many of the *Drosophila* hsps is up-regulated at the RNA level during normal aging (King and Tower, 1999; Landis et al., 2004; Pletcher et al., 2002; Wheeler et al., 1995; Wheeler et al., 1999; Zou et al., 2000), as well as at the protein level. The level of Hsp22 protein increase over 150-fold in old flies, and its induction during aging was observed in all tissues with particularly high-level expression in nervous and eye tissue (King and Tower, 1999). During aging of *Drosophila*, Hsp70 protein was induced 7- to 10-fold in thorax of old flies (36-38 day), relative to young flies (5-7 day) with the absence of heat shock, while no increase was observed in head or abdomen (Wheeler et al.,1995). In addition, RNA increase of *hsp22* and *hsp23* genes was observed in the thorax at the later time points (approximately after 30day), the increased expression was 8- to 10- fold for *hsp22* and 4- to 8-fold for *hsp23* between days 20 and 40 (Wheeler et al.,1995). However, most heat shock genes (*hsp22, hsp23, hsp26, hsp27, all hscs+hsp70*) in head or abdomen exhibited an accumulation of RNA at day 2 after eclosion, and then decreased with age (Wheeler et al., 1995). Several heat-shock genes were induced constitutively during aging of *Drosophila* apparently in response to accumulation of modified, partially denatured proteins (Niedzwiecki and Fleming, 1990). In the present study, during normal aging process of the control flies, the level of Hsp26, Hsp27 and Hsp83 specifically decreased in an age-dependent way. The long-lived Hsp22 over-expressing flies had a relatively less significant decline of these proteins.

Taken together, all of the results obtained here suggest that the beneficial effects of Hsp22 over-expression, specifically concerning the resistance to oxidative stress, the regulation of damaged protein and its influence upon the expression of other heat shock proteins, may contribute to the extended lifespan.

2.6 Acknowledgements

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Chapter 3. Isolation and identification of mitochondrial proteins of adult *Drosophila melanogaster*

3.1 Abstract

Alteration of the mitochondrial proteins has frequently been implicated in neurodegenerative diseases and in the aging process. Mitochondria have their own DNA, but the majority of mitochondrial proteins are encoded by nuclear DNA and the synthesized proteins in the cytoplasm are imported into the mitochondria. Therefore, the biogenesis and functioning of mitochondria are complex processes requiring the consideration of both nuclear and mitochondrial genetic systems.

In this study, we validated a mitochondrial isolation procedure in adult flies, and defined the *Drosophila* mitochondrial proteomes. To isolate intact mitochondria, we used differential centrifugation to obtain crude mitochondria. The soluble and insoluble mitochondrial fractions were then separated by treatment with the non-ionic detergent, Nonidet P-40 (NP-40). The proteins of each fraction (total, NP-40 soluble and NP-40 insoluble) were identified by LC-MS/MS ion trap mass spectrometer. The identified proteins were analyzed for their subcellular localizations using MITOPRED, a web server enabling prediction of nucleus-encoded mitochondrial proteins and Swiss-Prot/TrEMBL server, which directly indicates the protein localization by matching protein accession numbers.

139 proteins were identified in the crude mitochondria of adult flies. Among them, 90 proteins have a recognized mitochondrial localization, 28 have unknown subcellular location, and 22 have other various localizations. 35 of 41 proteins (85.4%) were in soluble mitochondrial fraction, and 20 of 41 proteins (48.8%) in insoluble mitochondrial fraction had mitochondrial localization.

3.2 Introduction

Defining proteome alterations during aging may indicate not only various metabolic changes including main targets of age-dependent functional decline but may also provide significant insight into the mechanisms of aging. A proteome analysis on whole cell extracts tends to miss relatively low-abundance proteins, as the number of proteins in a single cell of complex eukaryotes is estimated to approach 10,000 (Duncan and McConkey, 1982). One solution to this problem is to take advantage of the compartmentalization of the eukaryotic cell, and to analyze subcellular organelles. These can be isolated in good yield and the complexity of their protein composition is lower than that of whole cells.

Mitochondria are important organelles responsible for electron transport and oxidative phosphorylation in eukaryotic cells. They are closely being associated with aging or anti-aging processes. In addition to their primary role of intracellular energy production, these organelles sequester calcium and both generate and detoxify ROS (Nicholls, 2002). The generation of mitochondrial ROS is a consequence of oxidative phosphorylation. The controlled oxidation of NADH or FADH forms a redox potential by exported protons and imported electrons across the mitochondrial inner membrane. This potential energy in turn drives to phosphorylate ADP to ATP via the F1-F0 ATPase. During oxidative phosphorylation, the free radicals derived from several sites along the electron transport chain can directly react with oxygen or other electron acceptors and generate various types of reactive oxygen species.

Mitochondria contain 5-10% of the total proteins in cells (Alonso et al., 2005). Although the mitochondria have their own DNA and separated machinery of protein synthesis, the majority of the proteins required for functional mitochondrion are encoded from the nuclear DNA. Therefore, the proteins synthesized by the nucleus are imported into the mitochondrion only after transcription and translation (Sardiello *et al*., 2003); thus the biogenesis of mitochondria is a complex process requiring the contribution of both nuclear and mitochondrial genetic systems.

Hsp22 in *Drosophila*, the target protein for its role in longevity in the present study is known to localize in the mitochondrial matrix. Therefore, we suspect that the function of mitochondria in the long-lived flies over-expressing Hsp22 may be involved in the lifespan extension. Here, we validated a mitochondrial isolation procedure in adult flies, and defined the *Drosophila* mitochondrial proteomes.

3.3 Method

3.3.1 Isolation and purification of mitochondria from adult flies

Crude mitochondria were isolated from whole body of adult flies using a slightly modified protocol, which was applied for mice mitochondria isolation by Melov et al., (1999). The procedure was optimized with 200 flies per single isolation. Briefly, flies were placed on ice for 5 min, and then they were homogenized with 7ml of H-buffer (210mM mannitol, 70mM sucrose, 1mM EGTA, 5mM HEPES, pH 7.2) in a 15ml conical tissue grinder (Tissue Grinder System, VWR international, USA). Homogenization was performed for 30-60 seconds until a unified color of the extract was obtained. Subsequently, the homogenate was centrifuged at 1,000xg, 4°C, for 5min. The supernatant was transferred to a new, cold 15ml conical tube, and was re-centrifuged at 4°C, 1,000xg for 5min. Floating debris such as broken wings in the obtained supernatant was removed by passing through 100µm of nylon sieves (Cell Strainer REF 352360, BD Falcon, USA). Then, the supernatant was pelleted to yield a crude mitochondria fraction by centrifugation at 4°C, 8,800xg for 10min. For further purification, the mitochondria pellet was adjusted to a protein concentration of 5mg/ml in SEM buffer (250mM sucrose, 1mM EDTA, 10mM MOPS, pH 7.2) and loaded onto a sucrose gradient (1.5ml 15%, 1.5ml 23%, 4ml 32%, 1.5ml 60% sucrose in EM-buffer (1mM EDTA, 10mM MOPS, pH 7.2)). Then, an ultracentrifugation was performed for 1h at 134,000xg, 4°C, yielding highly pure mitochondria at the 60/32%-sucrose-interface. The total procedure is summarized in Fig. 3-1A (see page 68).

3.3.2 Confirmation of isolated mitochondria purity

Aliquots of the protein samples corresponding to an equivalent portion of the original homogenate were collected from all obtained fractions during mitochondria isolation and were separated by SDS-PAGE. The subsequent procedures of electrophoresis and Western blot were the same method previously shown in section 2.3.3. The purity of the mitochondria was examined by the Western blot using antibodies against proteins representative of various subcellular compartments. Antibodies against Cyt c (Pharmingen, 1/1000), Hsp22 (#36, 1/2500; Morrow et al., 2000) and Hsc70 (JLD3, 1/5000) were used for mitochondrial localization; Hsp83 (3E6, 1/100; Carbajal et al., 1990) for cytosol, Hsp27 (2C8, 1/100; Marin et al., 1993) for nucleus or cytosol, Histone H2B (1/20000) for nucleus. Peroxidase-

conjugated secondary antibodies of goat anti-rabbit or goat anti-mouse (1/10000, Jackson Immuno Research laboratories, West Grove, PA) were used. Chemiluminescent detection was performed using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instruction.

3.3.3 Proteins identification by LC-MS/MS analysis

The isolated mitochondrial pellet (approximately 10μg of protein) was resuspended in 10μl of 50mM NH_4HCO_3, and was treated with 2.5μl of 45mM DTT at 37℃ for 30min. The reduced mitochondrial protein was alkylated with 2.5μl of 100mM iodoacetamide in the dark at 25℃ for 20min and was diluted with 18μl of 50mM NH_4HCO_3 and 15μl CH_3CN. Subsequently, 2μl of 0.1μg/μl trypsin was added in and the protein was digested overnight at 37°C. The reaction was stopped by adding 5μl of 1% formic acid. After drying the solution using speed-vac evaporation, 10μl of 0.1% formic acid was added in. The digested sample was loaded onto a 75-μm internal diameter C18 picofrit column (New Objective). Peptides were eluted with a water-acetonitrile 0.1% formic acid gradient at a flow rate of about 200 nl/min. Tandem mass spectra were collected on an LTQ (ThermoFinnigan) quadrupole ion trap mass spectrometer with a nanospray interface following the parameters as shown below.

Parameters of LTQ ion trap mass spectrometer (ThermoFinnigan)

Digestion Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 2.0 Da
Fragment Mass Tolerance	: ± 0.5 Da
Max Missed Cleavages	: 2
Instrument type	: ESI-TRAP
Number of queries	: 7460

Resulting peptide spectra were interpreted using the MASCOT algorithm (Perkins et al., 1999) and searched against *Drosophila* proteins in the NCBI non-redundant protein database (http://www.ncbi.nlm.nih.gov/sites/entrez). Cysteine carboxamidomethylation was specified

as a static modification and methionine oxydation was allowed as a variable modification. The analysis was performed at the Proteomics Platform Quebec Genomics Center of the CHUL.

3.3.4 Prediction of mitochondrial proteins using proteomic database

The identified proteins were further analyzed for their subcellular localizations using Swiss-Prot/TrEMBL (ExPASy Proteomics Server, http://ca.expasy.org/) which directly indicate protein localization by matching protein accession numbers and MITOPRED web server (http://pathway.rit.albany.edu/~mitopred/) which enables to predict nucleus-encoded mitochondrial proteins. By using MITOPRED algorithm confidence of similarity in the peptide sequence to the mitochondrial proteins was scored for the identified proteins. Briefly, it is based on the differences in the Pfam (comprehensive database of protein domain family) domain occurrence patterns and amino acid compositional differences between mitochondrial and non-mitochondrial sequences (Guda et al., 2004a and b). For the prediction of mitochondrial protein, the confidence value was limited to over than 60%.

3.3.5 Separation of NP-40 soluble and insoluble mitochondrial fractions

The mitochondrial pellet obtained from flies were re-suspended with 1 volume of H-Buffer containing 0.5% Nonidet P-40, and incubated on ice for 1h to yield a mitochondrial soluble fraction (matrix and soluble intermembrane space (IMS)) and an insoluble membrane fraction (comprising both the outer membrane (MOM) and the inner membrane (MIM)). The incubated solution was centrifuged at 10,000 rpm for 10min, and then the supernatant was selected for a soluble fraction and the remaining pellet was designated as the insoluble fraction with briefly washing with H-Buffer without disturbing. All procedures were performed at 4°C. Proteins from both fractions were submitted to LC-MS/MS spectrometer analysis as for the crude mitochondrial pellet.

3.4 Results

3.4.1 Intact mitochondria are isolated in adult flies

The isolation of crude mitochondria from the total homogenate of adult flies was performed (Fig. 3.1 (A)) and subsequent purification was applied for the obtained mitochondrial pellet. In the interface between 60% and 32% of sucrose, mitochondrial fraction was clearly visualized as shown in Fig. 3-1 (B). The whole procedure of isolation and purification of mitochondria was analysed by Western blot using various subcellular marker proteins. Anti-Cyt c, anti-Hsp22 and anti-Hsc70 were utilized as reference markers for mitochondrial compartment, anti-Hsp83 was for cytosolic compartment, and anti-Histone H2B was for the nucleus. Anti-Hsp27 was able to indicate nucleus or cytosolic locations developmental stage specifically. Lane 6 and 7 in Fig. 3-1 (C) present clear signal of the antibodies indicating mitochondria location, while no signal of other antibodies indicating cytosol and nucleus. Therefore, mitochondria obtained in the crude mitochondrial fraction (CM) as well as in the sucrose gradient purified mitochondrial fraction (PM) are free of nucleus and cytosolic contaminants.

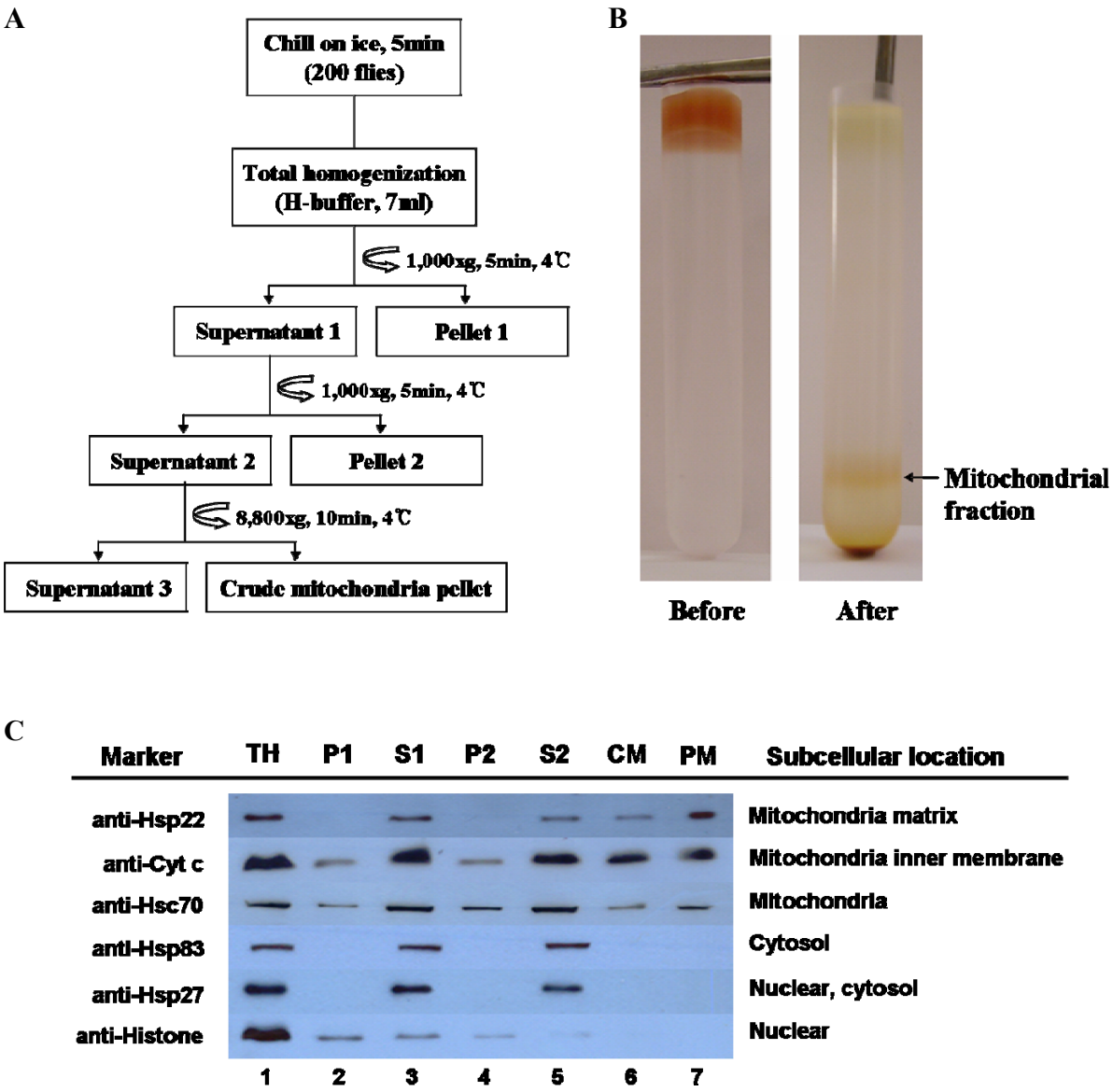

Fig. 3-1. Confirmation of purity of the isolated mitochondria in adult flies

A) Scheme of crude mitochondria fractionation, B) purification of the mitochondria through an ultracentrifugation in a sucrose gradient, C) confirmation of mitochondrial purity (of Hsp22 over-expressing flies) using marker antibodies indicating various subcellular locations. Crude mitochondria were isolated by a method using simple centrifugation. Purified mitochondria were obtained at the interference between 60% and 32% of sucrose after ultracentrifugation. TH; total homogenate, P1; pellet from TH, S1; supernatant from TH, P2; pellet from S1, S2; supernatant from S1, CM; crude mitochondria from S2, PM; purified mitochondria from CM.

3.4.2 Mitochondrial matrix proteins including Hsp22 are preferentially localized in the soluble fraction after NP-40 treatment

The crude mitochondria obtained from the Hsp22 over-expressing flies and the control flies were consequently treated with 0.5% NP-40, and then were separated to soluble and insoluble mitochondrial fractions. Using several marker proteins indicating the mitochondrial matrix localization, each fraction was examined. Hsp22 in the flies over-expressing Hsp22 (Hsp22+ flies) was present in the total and soluble mitochondrial fraction. Two other soluble proteins, Hsp60 (matrix) and Cyt c (intermembrane space) were also found in the total and soluble mitochondrial fractions. They especially showed a conserved amount of the protein between both fractions, although there was small fraction detected in the insoluble fraction in the case of Hsp60. Here, we once more confirmed that Hsp22 is present in the mitochondrial matrix by showing its clear localization in the soluble mitochondrial fraction.

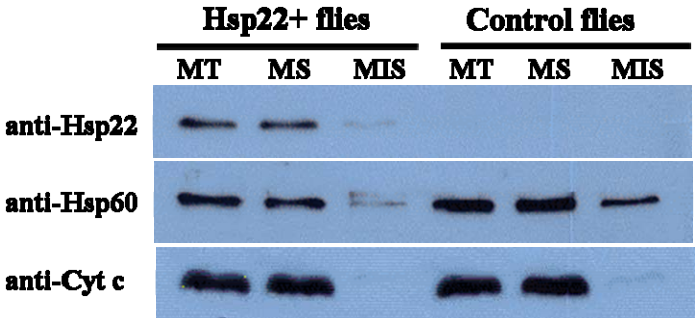

Fig. 3-2. Localization in the soluble mitochondrial fraction of the mitochondrial matrix proteins

MT; total mitochondria, MS; soluble fraction of mitochondria (inner membrane space and matrix; obtained from supernatant after treatment with 0.5% NP-40 for 30min), MIS; insoluble fraction of mitochondria (mitochondrial inner-, outer-membrane; obtained by centrifugation after treatment with 0.5% NP-40 for 30min).

3.4.3 Crude mitochondrial fraction contains a high proportion of known mitochondrial proteins

Identification of constitutive proteins of the crude mitochondrial pellet obtained from the control flies (*actin*-Gal4:TM3,*sb*) was performed by LC-MS/MS analysis. The resultant

protein queries revealed specific individual proteins with peptide sequence homologous to the *Drosophila melanogaster* proteome. The identified proteins were classified to their corresponding subcellular compartments as presented in Swiss-Prot/TrEMBL. Additionally, the confidence for mitochondrial proteins was predicted on a basis of similarity score by MITOPRED. As shown in Table 3-1, 139 proteins were reasonably identified in the crude mitochondrial fraction. Among them, 90 proteins had a link to the mitochondrial compartment, 27 proteins had no known subcellular localization and 22 proteins were related to non-mitochondrial compartments. Therefore, 64.7% of total identified proteins and 80.4% of the proteins having the known subcellular localization were known mitochondrial proteins. Most of the identified mitochondrial proteins (69 proteins) were located at inner membrane, 8 proteins in matrix, and 11 proteins non-specifically being related to mitochondrial membranes. Although parts of the identified proteins showed an unclear subcellular localization (19.4%) or non-mitochondrial identification (15.8%), their detection in the mitochondrial fraction seems to indicate the probability of their unrevealed mitochondrial localization or association with the mitochondrial compartment. For example, ‘Paramyosin’ known as a cytoplasmic myofibril protein however, showed 92.3% confidence for mitochondrial protein by MITOPRED. Secreted hormonal protein ‘neuropeptide-like 2’ also has 79.6% of confidence for mitochondrial protein.

Table 3-1. Proteins identified in mitochondria of adult *Drosophila melanogaster*

Accession numbers	ORF	Function	Protein molecular weight (Da)	MITOPRED confidence (subcellular localization)
Mitochondrion/mitochondrial inner membrane				
O18404	scu	3-hydroxyacyl-CoA dehydrogenase type-2	26887	99.0%
Q8T3P0	CG7145	1-pyrroline-5-carboxylate dehydrogenase activity	63693	99.0%
P17704	RpS17	40S ribosomal protein S17	15154	99.0%
P09180	RpL4	60S ribosomal protein L4	45026	100.0%
Q9VL70	yip2	Acetyl-CoA C-acyltransferase activity	41610	99.0%
Q9VAC1	CG7920	Acetyl-CoA metabolic process	51854	99.0%
Q9VU35	CG11267	Adenosinetriphosphatase, Chaperonin Cpn10 like	10998	100.0%
Q4QPQ0	Aldh	Aldehyde dehydrogenase	61908	99.0%
Q9VLC5	CG3752	Aldehyde dehydrogenase (NAD) activity	57019	99.0%
Q8SXQ1	CG9629	Aldehyde dehydrogenase (NAD) activity	58314	99.0%
Q24751	ATPsyn-beta	ATP synthase beta chain	23965	100.0%
Q05825	ATPsyn-beta	ATP synthase subunit beta	54091	100.0%
Q8T4C4	CG5389	ATP synthase subunit beta	67710	100.0%
Q6XIZ3	ATPsyn-d	ATP synthase, subunit d	20231	92.3%
Q9W141	CG4692	ATP synthase subunit f	12465	61.5%
Q6XHK8	CG12408	Calcium-binding EF-hand like	14114	61.5%
Q9V6U5	CG6543	Catalytic activity (similar to enoyl-CoA hydratase)	31582	99.0%
Q6XHF9	CG9920	Chaperonin Cpn10 like	11102	69.2%
Q8T477	kdn	Citrate synthetase	58199	99.0%
Q6XHL0	Scsalpha	CoA binding	18941	99.0%
Q7YZE9	CoVa	Cytochrome c oxidase polypeptide Va	16636	100.0%
Q6XHY7	CG11015	Cytochrome c oxidase, subunit Vb	13620	84.6%
Q9VRL0	CG4769	Cytochrome c1	33747	100.0%
Q9VIQ8	CG10664	Cytochrome-c oxidase	20501	100.0%
Q8IQW2	CG14235	Cytochrome-c oxidase activity	9286	100.0%
Q9VNW6	CG7470	Delta1-pyrroline-5-carboxylate synthetase activity	84091	99.0%
Q9VVL7	CG7430	Dihydrolipoyl dehydrogenase	53066	100.0%
Q9VM14	CG5261	Dihydrolipoyllysine-residue acetyltransferase	54234	100.0%
Q9VGQ1	CG5214	Dihydrolipoyllysine-residue succinyltransferase	49907	100.0%
Q8MR58	CG7834	Electron transfer flavoproteins	27327	100.0%
Q7KLW5	wal	Electron transfer flavoproteins, alpha subunit	35273	99.0%
Q8MLS0	CG13551	Enzyme inhibitor activity	9717	100.0%
Q8IRQ5	l(1)G0255	Fumarate hydratase	50442	100.0%
Q292E4	Dpse\GA14410	Fumarate reductase/succinate dehydrogenase	72268	99.0%
Q24269	l(3)03670	GAL4 enhancer trap line	24154	69.2%
AJFF1M (P20477)	Gs1	Glutamine synthetase 1	44396	69.2%
Q7K569	l(2)k05713	Glycerol-3-phosphate dehydrogenase	80406	84.6%
P29845	Hsc70-5	Heat shock 70 kDa protein cognate 5	74049	99.0%
Q9VD58	CG6439	Isocitrate dehydrogenase (NAD(+)).	40363	99.0%
Q8MT18	CG5028	Isocitrate dehydrogenase (NAD+) activity	44413	84.6%
Q9VWH4-2	l(1)G0156	Isocitric dehydrogenase [NAD] subunit alpha	38567	99.0%

Table 3-1. (continued)

Accession numbers	ORF	Function	Protein molecular weight (Da)	MITOPRED confidence (subcellular localization)
Q9V397	CG4389	Long-chain-3-hydroxyacyl-CoA dehydrogenase, long-chain-enoyl-CoA hydratase	84058	99.0%
Q9VEB1	CG7998	Malate dehydrogenase, L-lactate dehydrogenase	35300	84.6%
Q29AC6	GA19206	Malic enzyme	71840	84.6%
Q9VB69	Mdh	Malic enzyme	68593	84.6%
Q7KW39	CG17896	Malonate-semialdehyde dehydrogenase	55972	99.0%
Q9VIE8	Acon	Mitochondrial aconitase	85383	99.0%
Q7JUS9	CG9090	Mitochondrial carrier protein	40476	69.2%
Q9W125	CG3683	NADH dehydrogenase (ubiquinone)	19845	100.0%
Q9VWI0	CG12203	NADH dehydrogenase (ubiquinone)	20663	100.0%
Q9VXK7	CG9172	NADH dehydrogenase (ubiquinone)	24576	100.0%
Q9V8M5	CG15093	Probable 3-hydroxyisobutyrate dehydrogenase	33883	100.0%
Q9V9A9	l(2)01289	Protein disulfide isomerase activity	196579	69.2%
P24156	l(2)37Cc	Protein l(2)37Cc	30366	92.3%
Q9W2X6	l(1)G0230	Proton-exporting ATPase, ATP synthase	16709	100.0%
Q7KN97	CG1516	Pyruvate carboxylase activity	130862	99.0%
Q7K5K3,Q8IGJ4	CG11876	Pyruvate dehydrogenase (acetyl-transferring)	39333	99.0%
Q7KVX1,Q9W4H6	l(1)G0334	Pyruvate dehydrogenase (acetyl-transferring)	43819	100.0%
Q9VTP4	RpL10Ab	Ribosomal protein L10Ab	24274	100.0%
Q9W1B9	RpL12	Ribosomal protein L12	17673	100.0%
Q9VVU1	CG3902	Short-branched-chain-acyl-CoA dehydrogenase activity	45366	100.0%
Q9VJ43	ScpX	Sterol carrier protein X-related thiolase	59007	69.2%
Q9VHJ8	CG11963	Succinate-CoA ligase (ADP-forming) activity	54807	100.0%
Q95U38	CG11963	Succinate-CoA ligase (ADP-forming)	54895	100.0%
Q6XIS3	Sod2	Superoxide dismutase 2	14661	76.9%
P36188	wupA	Troponin I, development and maintenance of muscle and nervous system	30064	61.5%
Q9VV75	CG4169	Ubiquinol-cytochrome-c reductase	45397	99.0%
Q9VFF0	CG3731	Ubiquinol-cytochrome-c reductase, mitochondrial processing peptidase	51856	92.3%
Q8SXT2	Vha68-2	Vacuolar ATP synthase catalytic subunit A isoform 2	68275	100.0%
Mitochondrial matrix				
Q8MYV0	CG7433	4-aminobutyrate transaminase activity	54530	99.0%
Q8IQQ0	Nc73EF	Alpha-ketoglutarate dehydrogenase	113608	100.0%
P84029	Cyt-c-p	Cytochrome c-2	11717	100.0%
P54385	Gdh	Glutamate dehydrogenase	62520	100.0%
Q6NR71	Hsp60	Heat shock protein 60	60778	99.0%
Q9VSA3	CG12262	Probable medium-chain specific acyl-CoA dehydrogenase	45854	100.0%
Q9VAP7	CG11876	Pyruvate dehydrogenase (acetyl-transferring)	39351	99.0%
Q7YU05	l(1)G0334	Pyruvate dehydrogenase (acetyl-transferring)	35894	100.0%

Table 3-1. (continued)

Accession numbers	ORF	Function	Protein molecular weight (Da)	MITOPRED confidence (subcellular localization)
Mitochondrial membrane				
Q26365	sesB	ADP/ATP translocase	34197	--
Q24407	ATPsyn-Cf6	ATP synthase coupling factor 6	11918	100.0%
Q24439	Oscp	ATP synthase oligomycin sensitivity conferral protein	22405	100.0%
P35381	blw	ATP synthase subunit alpha	59405	100.0%
Q94516	ATPsyn-b	ATP synthase subunit b	27340	100.0%
Q24251	ATPsyn-d	ATP synthase subunit d	20182	100.0%
O01666	ATPsyn-gamma	ATP synthase subunit gamma	32854	100.0%
P00408	CoII	Cytochrome c oxidase subunit 2	26379	100.0%
Q94514	CoVa	Cytochrome c oxidase subunit 5A	16384	100.0%
Q94511	ND75	NADH-ubiquinone oxidoreductase 75 kDa subunit	78613	100.0%
Q94920	porin	Voltage-dependent anion-selective channel	30532	100.0%
Others				
Q9VS34	CG8615	60S ribosomal protein L18	21664	Cytoplasm
P02572	Act42A	Actin-42A	41824	Cytoplasm
Q6XHG6	Cam	Calmodulin; calcium ion binding (EF-Hand_type like)	16494	Cytoplasm
Q8IPX8	Eno	Enolase	54310	Cytoplasm
P07487	Gapdh2	Glyceraldehyde-3-phosphate dehydrogenase 2, glycolysis	35351	Cytoplasm
Q8INZ9	Mhc	Myosin heavy chain	221464	Cytoplasm
Q8SXN3	Act57B	Actin-57B	41817	Cytoplasm, cytoskeleton
P02574	Act79B	Actin-79B	41769	Cytoplasm, cytoskeleton
P05661-17,P05661-22	Mhc	Myosin heavy chain, muscle contraction	224479	Cytoplasm, myofibril
P05661-2	Mhc	Myosin heavy chain, muscle contraction	224397	Cytoplasm, myofibril
P35415	Prm	Paramyosin, long form, a major structural component of many thick filaments isolated from invertebrate muscles	102322	92.3%, (Cytoplasm, myofibril)
P53777	Mlp60A	Muscle LIM protein 1	9940	Cytoplasm, nucleus
Q8IPY3	CG4233	Aspartate aminotransferase	48172	Cytoplasmic, mitochondrial & chloroplastic isozymes
Q29HE5	Dpse\GA17988	GA17988-PA , similarity to heat shock protein 70 family	72289	Endoplasmic reticulum lumen
JN0666	Hsc70-3	Heat shock 70 kDa protein cognate 3	72261	Endoplasmic reticulum lumen
Q9TWZ1	Pdi	Thioredoxin domains similarity	55372	Endoplasmic reticulum lumen
P54399	Pdi	Protein disulfide-isomerase	55764	Endoplasmic reticulum lumen

Table 3-1. (continued)

Accession numbers	ORF	Function	Protein molecular weight (Da)	MITOPRED confidence (subcellular localization)
P22700	Ca-P60A	Calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type	111686	Endoplasmic reticulum membrane
Q95029	Cp1	Cathepsin L (cysteine proteinase 1)	37971	Lysosomal
P13607	Atpalpha	Sodium/potassium-transporting ATPase alpha chain (Na+/K+ ATPase)	115591	Membrane
P84040	His4r, His4	Histone H4	11363	Nucleus
P17336	Cat	Catalase	57150	Peroxisomal
Q9VU58	Nplp2	Neuropeptide-like 2, neuropeptide hormone activity	9394	76.9%, (secreted)
Q9V496	RfaBp	Retinoid- and fatty acid-binding glycoprotein	372673	Secreted
Q6XHZ7	14-3-3zeta	14-3-3 zeta protein (domain specific binding)	20456	Unknown
Q9GN94	Adh	Alcohol dehydrogenase	27743	Unknown
Q8IQC0	Argk	Arginine kinase	47889	Unknown
Q6NNE3	CG2184	Calcium ion binding (EF-Hand_type like)	23185	Unknown
A1ZA67	Strn-Mlck	Calcium/calmodulin-dependent protein kinase	496991	Unknown
A1Z7Z4,Q7K2P3,Q8IGD2	CG1648	CG1648-PB, isoform B	24201	Unknown
Q04691	Fbp1	Fat-body protein 1	119646	Unknown
P35554,Q71D79	fln	Flightin, regulation of flight muscles contraction	20638	Unknown
Q7KRZ0	Ald	Fructose-bisphosphate aldolase	43243	Unknown
Q9VAY2	Gp93	Glycoprotein 93	90238	Unknown
Q0E924	l(2)03709	lethal (2) 03709	26859	Unknown
P18432	Mlc2	Myosin regulatory light chain 2	23697	Unknown
Q9VQR2	Pdsw	NADH dehydrogenase (ubiquinone)	18914	Unknown
Q8SYJ2	CG32230	NADH dehydrogenase activity	9434	Unknown
Q6XHE3	CG7217	Oxidoreductase activity (Redoxin like)	16339	Unknown
Q7K084	Obp44a	Pheromone/general odorant binding protein, PBP/GOBP	15843	Unknown
Q8IMH4	Sap-r	Saposin-like	97617	Unknown
Q6NN28	Tm1	Tropomyosin-1	32757	Unknown
P49455	Tm1	Tropomyosin-1, isoforms 33/34	54567	Unknown
Q7KSI0	Tm1	Tropomyosin-1, isoforms 33/34	54585	Unknown
Q7KSI1	Tm1	Tropomyosin-1, isoforms 9A/A/B	32745	Unknown
Q9VF96	Tm1	Tropomyosin-1, isoforms 9A/A/B	39325	Unknown
Q9VF97	Tm1	Tropomyosin-1, isoforms 9A/A/B	29297	Unknown
P09491	Tm2	Tropomyosin-2, plays a central role in the calcium dependent regulation of muscle contraction.	32788	Unknown
Q6T2Y6	TpnT	Troponin T	44901	Unknown
Q6IMD2	TpnT	Troponin T isoform 5	44901	Unknown
P19351-3	up	Troponin T, skeletal muscle	47471	Unknown

3.4.4 Proteins in the soluble fraction of crude mitochondria preferentially reveal mitochondrial functions involved in the oxidative phosphorylation and TCA cycle

The crude mitochondria isolated in adult flies (control flies, *actin*-Gal4:TM3*,sb*) were treated by H-buffer containing NP-40, and separated into a soluble fraction (matrix and inter membrane space) and an insoluble membrane fraction. Identification of the proteins in the soluble fraction was performed by LC-MS/MS analysis, and 41 proteins (present only in the soluble fraction and not in the insoluble fraction) were detected as shown in Table 3-2. Among them, 35 proteins (85.4% of 41 proteins) were already known mitochondrial proteins and remarkably had their corresponding localization of the mitochondrion, mitochondrial inner/outer membrane, and mitochondrial matrix. More frequently detected protein peptides showed a homology to ATP synthase subunits, cytochrome c, NADH dehydrogenase, and several enzymes involved in TCA cycle. Moreover, these proteins were frequently detected in the total fraction of crude mitochondria as indicated by the number of matched peptides. Therefore, major constitution of mitochondrial protein consists of proteins present in the soluble fraction.

On the other hand, the protein ‘glyceraldehyde-3-phosphate dehydrogenase 2’ identified by peptide O04414 has been known to localize in cytoplasm. The sequence of O04414 peptide, however showed 99.0% of similarity confidence for mitochondrial protein by MITOPRED. Furthermore, the peptide Q9VU58 for ‘neuropeptide-like 2’ was present in the soluble fraction (Table 3-2), in addition to its presence in the total fraction of crude mitochondria (Table 3-1). Therefore, these results of the involvements in mitochondrial fraction (total and soluble) and 76.9% confidence for mitochondrial protein are suggestive for high potential to be a novel mitochondrial protein as well as being a secreted protein. More notably, protein peptides of unknown localization, such as ‘sarcoplasmic calcium-binding protein 1’, ‘arginine kinase’, ‘lethal(2)03709’ and ‘NADH dehydrogenase homologous peptide’ in the soluble mitochondrial fraction may also be considered to have a putative mitochondrial localization or association.

Besides, muscle LIM protein Mlp84B that is known to localize in cytoplasm or nucleus was present in the soluble fraction. The conserved mitochondrial proteins, such as alpha-ketoglutarate dehydrogenase, Hsc70-5, Hsp60, malate (L-lactate) dehydrogenase,

mitochondrial aconitase and ubiquinol cytochrome-c reductase were detected with higher number of peptides.

3.4.5 Proteins in the insoluble fraction of crude mitochondria are involved in certain cytoplasmic functions as well as known mitochondrial functions

After the treatment of the crude mitochondria with H-buffer containing NP-40, the yielded insoluble fraction was applied for LC-MS/MS analysis following the same procedure as the soluble fraction. The 41 proteins specifically identified in the insoluble fraction (not in the soluble fraction) are shown in Table 3-3. 48.8% of the proteins had a known mitochondrial localization and they were related to large and small ribosomal proteins, cuticular protein 64Ab, enoyl-CoA hydratase, fatty acid synthase, lectin type C, pyruvate carboxylase, cytochrome c oxidase polypeptide VIc and some unknown functions.

On the other hand, several peptides (Q7K5K9, Q961W5, Q500X4, Q7K2S9, Q9VSM7, Q9VSM6, Q3YMW1 and Q95R35) on ‘leucyl aminopeptidase’ known as cytoplasmic localization were frequently detected in the insoluble fraction. Secreted proteins such as ‘ejaculatory bulb-specific protein’ and ‘glutactin’ were present in the insoluble mitochondrial fraction. The homologous peptides indicating ‘collagen alpha-1 (IV) chain’, ‘calcium/calmodulin-dependent myosin kinase’, ‘glutathione S-transferase S1’, ‘salivary glue protein Sgs-5’ and ‘tubulin beta-2 chain’ were identified in the insoluble fraction of crude mitochondria indicating putative mitochondrial localization or association although they presented unknown localization.

Table 3-2. Proteins identified in the soluble mitochondrial fraction

Accession numbers	ORF	Function	Molecular weight (Da)	Subcellular localization	MITOPRED confidence	Number of peptides		
						Total	Insoluble	soluble
Q8IQQ0, Q9VVC5	Nc73EF	Alpha-ketoglutarate dehydrogenase	113608	Mitochondrial	100.0%	5	0	6
P48610, P48610-2	Argk	Arginine kinase	39849	Unknown	--	1	0	2
Q24439	Oscp	ATP synthase oligomycin sensitivity conferral protein	22405	Mitochondrion inner membrane	100.0%	7	0	4
Q94516	ATPsyn-b	ATP synthase subunit b	27340	Mitochondrion inner membrane	100.0%	3	0	2
Q24251	ATPsyn-d	ATP synthase subunit d	20182	Mitochondrion inner membrane	100.0%	6	0	5
Q9W141	CG4692	ATP synthase subunit f	12465	Mitochondrion	61.5%	4	0	2
Q9VRL0	CG4769	Cytochrome c1	33747	Mitochondrial	100.0%	5	0	2
P84029	Cyt-c-p	Cytochrome c-2	11717	Mitochondrial matrix	100.0%	5	0	5
Q9VVL7	CG7430	Dihydrolipoyl dehydrogenase	53066	Mitochondrial	100.0%	6	0	7
Q9VM14	CG5261	Dihydrolipoyllysine-residue acetyltransferase	54234	Mitochondrial	100.0%	5	0	3
Q7JR58	CG6543	Enoyl-CoA hydratase	31564	Mitochondrial	99.0%	1	0	2
Q8IRQ5	l(1)G0255	Fumarate hydratase	50442	Mitochondrial	100.0%	2	0	2
Q292E4	Dpse\GA14410	Fumarate reductase/succinate dehydrogenase	72268	Mitochondrial	99.0%	2	0	3
P54385	Gdh	Glutamate dehydrogenase	62520	Mitochondrial matrix	100.0%	4	0	4
O44104	Gapdh2	Glyceraldehyde-3-phosphate dehydrogenase 2, glycolysis	35294	Cytoplasm	99.0%	0	0	3
Q7K569	l(2)k05713	Glycerol-3-phosphate dehydrogenase	80406	Mitochondrial	84.6%	4	0	3
P29845	Hsc70-5	Heat shock 70 kDa protein cognate 5	74049	Mitochondrial	99.0%	5	0	4
O02649, Q6NR71	Hsp60	Heat shock protein 60	60778	Mitochondrial matrix	99.0%	4	0	5
Q9VD58	CG6439	Isocitrate dehydrogenase (NAD+) activity	40363	Mitochondrial	99.0%	2	0	2
Q8MT18	CG5028	Isocitrate dehydrogenase (NAD+) activity	44413	Mitochondrial	84.6%	2	0	3
Q9VWH4-2	l(1)G0156	Isocitric dehydrogenase [NAD] subunit alpha	38567	Mitochondrial	99.0%	4	0	4
Q29MI0	GA10231	Legume lectin	157188	Mitochondrial	99.0%	1	0	2
Q0E924	l(2)03709	lethal (2) 03709	26859	Unknown	--	2	0	2
Q9VIE8	Acon	Mitochondrial aconitase	85383	Mitochondrial	99.0%	15	0	11

Table 3-2. (continued)

Accession numbers	ORF	Function	Molecular weight (Da)	Subcellular localization	MITOPRED confidence	Number of peptides		
						Total	Insoluble	soluble
Q24400	Mlp84B	Muscle LIM protein Mlp84B	53507	Cytoplasm, nucleus.	--	0	0	2
Q8SY88, Q9W125	CG3683	NADH dehydrogenase (ubiquinone)	19845	Mitochondrial	100.0%	2	0	2
Q9VEB1	CG7998	Malate dehydrogenase, L-lactate dehydrogenase	35300	Mitochondrial	84.6%	9	0	6
Q9VXK7	CG9172	NADH dehydrogenase (ubiquinone).	24576	Mitochondrial	100.0%	2	0	3
Q9VTU2	l(3)neo18	NADH dehydrogenase (ubiquinone).	22012	Mitochondrial	99.0%	1	0	2
A0AQ24, Q9VQR2	Pdsw	NADH dehydrogenase (ubiquinone)-like pdsw subunit	18914	Unknown	--	2	0	2
Q9V4E0	CG1970	NADH dehydrogenase (ubiquinone).	52904	Mitochondrial	100.0%	0	0	2
Q9VMI3	CG9140	NADH dehydrogenase (ubiquinone).	51813	Mitochondrial	100.0%	1	0	2
Q94511	ND75	NADH-ubiquinone oxidoreductase 75 kDa subunit	78613	Mitochondrion inner membrane	100.0%	4	0	3
Q9VU58	Nplp2	Neuropeptide-like 2, neuropeptide hormone activity	9394	Secreted	76.9%	2	0	2
P24156	l(2)37Cc	Protein l(2)37Cc	30366	Mitochondrial	92.3%	2	0	3
Q7K5K3, Q8IGJ4	CG11876	Pyruvate dehydrogenase (acetyl-transferring)	39333	Mitochondrial	99.0%	6	0	3
Q7KVX1, Q9W4H6	l(1)G0334	Pyruvate dehydrogenase (acetyl-transferring)	43819	Mitochondrial	100.0%	4	0	3
Q6XHW1, Q8MSI2	Scp1	Sarcoplasmic calcium-binding protein 1	21164	Unknown	--	1	0	2
P13607, P13607-3, P13607-4, P13607-5, P13607-6, P13607-7	Atpalpha	Sodium/potassium-transporting ATPase subunit alpha	115591	Membrane, multi-pass membrane protein	--	4	0	4
O77466, Q9W1H8	CG4581	Thiolase	50602	Mitochondrial	99.0%	1	0	2
Q9VFF0	CG3731	Ubiquinol--cytochrome-c reductase, mitochondrial processing peptidase.	51856	Mitochondrial	92.3%	5	0	7

Table 3-3. Proteins identified in the insoluble mitochondrial fraction

Accession numbers	ORF	Function	Molecular weight (Da)	Subcellular localization	MITOPRED confidence	Number of peptides		
						Total	insoluble	soluble
Q8T3U2	RpS23	40S ribosomal protein S23	15998	Mitochondrial	100.0%	0	3	0
Q290H1, Q9W229	RpS24	40S ribosomal protein S24	14986	Mitochondrial	99.0%	0	2	0
P13008	RpS26	40S ribosomal protein S26	13248	Mitochondrial	92.3%	0	2	0
P41042	RpS4	40S ribosomal protein S4	29117	Mitochondrial	99.0%	0	3	0
P55935, Q29EE3	RpS9	40S ribosomal protein S9	22606	Unknown	--	0	3	0
Q9VJY6	RpL24	60S ribosomal protein L24	17502	Unknown	--	0	3	0
P18091, P18091-2, P18091-3, P18091-4, P18091-5	Actn	Alpha-actinin, sarcomeric	107004	Unknown	--	0	12	0
Q7KQP5	bt	Calcium/calmodulin-dependent myosin light chain kinase	968185	Unknown	--	0	6	0
Q7JQU6	CG30376	CG30376-PA	26385	Mitochondrial	92.3%	0	2	0
Q8I0F1	CG31538	CG31538-PA	63782	Mitochondrial	92.3%	0	2	0
Q8T0U2	CG5089	CG5089-PA	51727	Mitochondrial	92.3%	0	2	0
UPI000007EAD0, P08120	Cg25C	Collagen alpha-1(IV) chain	174285	Unknown	--	0	3	0
Q9VZG1	Cpr64Ab	Cuticular protein 64Ab	15397	Mitochondrial	61.5%	0	2	0
Q7YZE1, Q9VMS1	CG14235	Cytochrome c oxidase polypeptide VIc	8292	Mitochondrial	100.0%	1	2	0
Q9W4Y3	trol	EGF-like laminin	513388	Extracellular matrix	69.2%	0	4	0
Q9U6L5	Peb	Ejaculatory bulb-specific protein 1	37803	Secreted	--	0	4	0
Q23982	PebII	Ejaculatory bulb-specific protein 2	7168	Secreted	--	1	2	0
Q9VXI1	CG9914	Enoyl-CoA hydratase	34769	Mitochondrial	76.9%	0	2	0
Q9VQL7	CG3523	Fatty-acid synthase.	266429	Mitochondrial	100.0%	0	2	0
P33438	Glt	Glutactin	118731	Secreted	--	0	2	0
P41043	GstS1	Glutathione S-transferase S1	27595	Unknown	--	1	2	0
P02283	His2B	Histone H2B	13678	Nucleus	84.6%	0	4	0
Q4U3Q2, Q9VLW1	lectin-28C	Lectin type C	29912	Mitochondrial	61.5%	0	2	0
Q7K5K9	CG4439	Leucyl aminopeptidase	57761	Cytoplasm	--	0	5	0
Q961W5	CG8040	Leucyl aminopeptidase	57684	Cytoplasm	--	0	4	0
Q500X4	CG13340	Leucyl aminopeptidase, manganese ion binding, proteolysis	57420	Cytoplasm	--	0	7	0
Q9VSM7	CG32351	Leucyl aminopeptidase, manganese ion binding, proteolysis	61373	Cytoplasm	--	1	4	0

Table 3-3. (continued)

Accession numbers	ORF	Function	Molecular weight (Da)	Subcellular localization	MITOPRED confidence	Number of peptides		
						Total	insoluble	soluble
Q9VSM6	CG6372	Leucyl aminopeptidase, manganese ion binding, proteolysis	61496	Cytoplasm	--	1	10	0
Q7K2S9	CG4750	Leucyl aminopeptidase, manganese ion binding, proteolysis	56705	Cytoplasm	--	0	8	0
Q3YMW1, Q95R35	CG8040	Leucyl aminopeptidase.	57492	Cytoplasm	--	0	2	0
P05661-17, P05661-22	Mhc	Myosin heavy chain, muscle contraction	224479	Cytoplasm, myofibril.	--	2	121	0
Q9VGX3	fau	Protein anoxia up-regulated	68623	Mitochondrial	84.6%	1	5	0
Q8IMW8	BEST:GH24664	Protein unknown function DUF1431, cysteine-rich	26076	Mitochondrial	69.2%	0	3	0
Q0E9E2, Q7KN97	CG1516	Pyruvate carboxylase	132658	Mitochondrial	99.0%	1	2	0
Q29DK7, Q9VVU2	RpL26	Ribosomal protein L26	17295	Mitochondrial	99.0%	0	2	0
Q6XHX3, Q8MLY8	GE23892	Ribosomal protein S8	23746	Mitochondrial	84.6%	0	2	0
P07701	Sgs5	Salivary glue protein Sgs-5	18802	Unknown	--	0	2	0
Q00963	beta-Spec	Spectrin beta chain	265726	Cytoplasm, cytoskeleton	--	0	2	0
Q9I7U4-3	sls	Titin	2034042	Cytoplasm, nucleus	61.5%	0	10	0
P61857	TubB85D	Tubulin beta-2 chain	49852	Unknown	--	0	7	0
Q9VMV5	vkg	Type 4 procollagen, C-terminal repeat	193760	Unknown	--	0	2	0

3.5 Discussion

The aim of this study was to validate the methodology of mitochondrial isolation in the adult flies for the purpose of aging study, and to assess the purifity of the obtained mitochondrial fraction. The crude mitochondrial fraction obtained using repeated fractionation by differential centrifugation, as well as the additionally purified mitochondrial fraction via sucrose gradient indicated no nucleus and cytosolic contamination (Fig. 3-1, C). In the last years, much effort has been spent on the extensive purification of mitochondria as starting material for mitochondrial proteome studies which aimed to identify the entire inventory of truly mitochondria-localized proteins (Reifschneider et al., 2006). An extended purification procedure, however may damage mitochondria, especially those that were already impaired *in vivo* like aged ones. This may cause the loss of mitochondria or parts of their proteome due to disruption and leaking out (Nagai et al., 2000), and/or may affect the stability of protein-protein interactions during subsequent analysis (Krause et al., 2004, Reifschneider et al., 2006). Therefore, in this study, we used crude mitochondria for further experiments on the *Drosophila* mitochondrial proteome.

The mitochondrial matrix proteins (Hsp22, Hsp60 and Cyt c) which were examined in the SDS gel (Fig. 3-2) or through the LC-MS/MS analysis (Table 3-2) demonstrated very consistent result of their preferential presence in the soluble fraction. Some proteins of unknown localization were identified in both total and soluble fractions of the crude mitochondria. They were arginine kinase, lethal(2)03709, NADH dehydrogenase-like peptides (Pdsw subunit), sarcoplasmic calcium-binding protein 1. As the soluble fraction of the mitochondria indicates the inner membrane space and matrix, it seems that these proteins of unknown localization have higher possibility of being novel mitochondrial proteins. Interestingly, the characterization of the *Drosophila melanogaster* mitochondrial proteome by Alonso et al., (2005) also identified the inclusion of lethal(2)03709, NADH dehydrogenase-like peptides (Pdsw subunit) in the purified intact mitochondria. Secreted protein (neuropeptide-like 2) and cytoplasmic protein (glyceraldehyde-3-phosphate dehydrogenase 2) were found in the mitochondrial soluble fraction, and their mitochondrial localization was supported by the MITOPRED confidence of 99.0% and 76.9% respectively.

On the other hand, the insoluble fraction of the mitochondria contained a higher proportion of proteins having unknown or non-mitochondrial localization compared to that of soluble fraction. As 'glutathione S-transferase S1' was detected in both total and insoluble fraction of mitochondria, this protein seems to have some association with mitochondrial membrane perhaps for a function in cellular detoxification. Some secreted proteins like 'ejaculatory bulb-specific protein 1 and 2' and 'glutactin' were present in the insoluble fraction. Notably, the insoluble mitochondrial fraction contained proteins such as 'myosin heavy chain', 'alpha-actinin', 'calcium/calmodulin-dependent myosin light chain kinase', 'titin', 'leucyl aminopeptidase' and 'tubulin beta-2 chain', having relatively high number of peptides detection in the LC-MS/MS analysis. For instance, very specifically, the 'myosin heavy chain' had 121 peptides detected. Besides, certain proteins like 'EGF-like laminin' (69.2%) known as extracellular matrix localization, 'histone H2B' (84.6%) known as nuclear localization and 'titin' (61.5%) known as cytoplasmic or nuclear localization were predicted to be putative mitochondrial proteins by MITOPRED confidence indicated in a parenthesis.

There are some reports indicating that the mitochondria seem to have certain association with other cell compartments. The mitochondria of different mammalian cells communicate with other cell compartments such as the nucleus (Dzeja et al., 2002), endoplasmic/sarcoplasmic reticulum (Achleitner et al.; 1999, Simpson and Russell, 1997; Rizzuto et al. 1998), golgi (Dolman et al., 2005), cytosolic proteins (Giegé et al., 2003) and the cytoskeleton (Bereiter-Hahn and Voth, 1994, Fuchs et al., 2002) by close physical contacts. These associations between mitochondria and other compartments are precisely regulated. Furthermore, it appears that the mitochondria as the main ATP generator are nearby connected with ATP-consuming sites of other cell compartments (Dzeja et al., 2002; Dzeja and Terzic, 2003; Andrienko et al., 2003). Otherwise, the tight coordination of signaling event seems to result in the connection with the mitochondria (Duchen, 1999; Simpson and Russell, 1997; Rizzuto et al., 1998; Dolman et al., 2005; Hajnoczky et al., 2002; Walter and Hajnoczky, 2005; Michelangeli et al., 2005; Frieden et al., 2005). This probable communication between the mitochondria and other compartments was exemplified in the present study by the occurrence of major non-mitochondrial ATP-consuming proteins such as sodium/potassium-transporting ATPase subunit alpha, myosin heavy chain involved in muscle contraction and leucyl aminopeptidase indicating proteolysis activity, especially in the insoluble fraction containing the mitochondrial membranes. Although we still can not rule out the possible interactions between mitochondria

and other compartments, the presence of these proteins in the mitochondrial fraction could also arise from a contamination with other organells such as lysosome which are widely known as mitochondrial contaminants during preparing mitochondria.

In summary, we have clearly demonstrated that the mitochondrial localization of Hsp22 in the adult fly *Drosophila melanogaster* is a general phenomenon. The crude mitochondria obtained from the adult flies were intact and had sufficient purity for the subsequent experiments. Nevertheless, the involvement of several proteins having unknown or non-mitochondrial localizations in the mitochondrial fractions may indicate the presence of novel mitochondrial proteins as well as the specific interaction between mitochondrial proteins and proteins from other cell compartments.

3.6 Acknowledgements

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Chapter 4. Mitochondrial proteome and electron transporting chain activity in the long-lived flies over-expressing Hsp22

4.1 Abstract

In *Drosophila melanogaster*, over-expression of the small heat shock protein DmHsp22 that is localized in mitochondria matrix has shown beneficial effect on longevity. However, the detailed mechanisms responsible for lifespan extension are not clear. As differences in mitochondrial proteome may be involved in the longevity mechanism, the relative abundance in mitochondrial proteome was compared between the flies over-expressing Hsp22 by activating GAL4/UAS system and those of normal-expressing Hsp22 using a proprietary double-labeling technique (ProteoSys).

Both differently labeled mitochondria samples were separated on the same 2D-PAGE gel. The spots showing significant differences were selected, and then the individual protein spots were identified by MALDI-TOF analysis. From this, 21 spots of higher abundance and 4 spots of lower abundance in long-lived Hsp22 over-expressing flies have been identified. Functions of the proteins having homology to the identified spots are involved in ATP synthase/ATP synthesis coupled electron transport, TCA cycle, fatty acid beta oxidation, voltage-dependent anion-selective channel, amino acid metabolism, and anti-oxidative response. Especially, the ubiquitous aspartic protease, Cathepsin D, which is involved in autophagic salivary gland cell death during *Drosophila* metamorphosis was relatively the most abundant protein in Hsp22 over-expressing flies. On the other hand, L-malate dehydrogenase showed a possibility of posttranslational modification by presenting both higher and lower spot-abundance. The alteration of these specific enzymes in long-lived Hsp22 over-expressing flies may indicate their involvement in mechanisms to prolong lifespan.

4.2 Introduction

As indicated in the free radical theory of aging by Harman (1956), the deleterious effects of free radicals produced during the mitochondrial energy metabolism may be a mitochondria-specific reason of aging. Mitochondria have a central role in energy production through the numerous catabolic and anabolic metabolisms (Scheffler, 2001) such as oxidative phosphorylation, TCA cycle and β-oxidation of fatty acid. They also play crucial roles in cellular signaling pathways such as intracellular calcium homeostasis (Duchen, 1999) as well as programmed cell death (Green and Reed, 1998). Therefore, alterations in mitochondrial proteome are expected to reflect main targets which are associated with aging or anti-aging process. Groebe et al. (2007) found that ATP synthase was affected by age-dependent processes, underlying plausible posttranslational modifications through a comparative mitochondrial proteomes across various species. Moreover, comparative proteomic approach from aged monkey hearts presented gender-specific alteration in key enzymes in glycolysis, glucose oxidation, TCA cycle, and electron transport chain (complexes III-V), showing age-dependent decrease in males but not in females (Yan et al., 2004).

The vast majority (approximately 90%) of cellular ROS are generated in mitochondria as a consequence of oxidative phosphorylation (Lesnefsky and Hoppel, 2003, Balaban et al., 2005). Therefore, mitochondria appear to be a key target of oxidative damage during aging. In *Drosophila*, it has been reported that the amount of the mitochondrial DNA (mtDNA) representing roughly 1% of the total DNA remained fairly constant at all ages and a high stability and integrity of the mitochondrial DNA (mtDNA) were revealed (Calleja et al., 1993). A dramatic decline in mitochondrial transcripts such as 16 S ribosomal RNA (16SrRNA), cytochrome c oxidase, cytochrome b, and beta H(+)-ATP synthase subunit, however was detected during aging (Calleja et al., 1993). Furthermore, although no statistically significant influences of age on activities of complexes I and II or citrate synthase were observed, declines were found to be 40% in complex IV cytochrome c oxidase activity and 15% in ATP abundance from 2 to 45 days post-eclosion (Schwarze et al., 1998). Oxidatively damaged (carbonylated) forms of some mitochondrial proteins, like aconitase and adenine nucleotide transporter (ANT-C) have been reported to accumulate during aging

(Yan et al., 1997, Yan and Sohal, 1998) and structurally abnormal mitochondria are found to accumulate in various *Drosophila* tissues (Fleming et al., 1985, Miquel et al., 1976).

Drosophila is a well-established model organism in cellular and developmental biology. In addition, mitochondrial alterations in *Drosophila* mimic some human diseases which are related to mitochondrial dysfunction such as diabetes, obesity, cancer, aging, neurodegeneration, and cardiomyopathy (Scharfe et al., 1999). From the previously shown data in Chapter 2, over-expression of Hsp22 in mitochondria of *Drosophila melanogaster* resulted in extended lifespan and increased resistance to oxidative stress. Therefore, revealing the differential proteome in the long-lived Hsp22+ flies may indicate the key factors related with the possible mechanisms of longevity by over-expression of Hsp22.

4.3 Method

4.3.1 Flies and sample preparation

The flies were prepared as previously described in 2.3.1. Among the siblings, approximately 1000~1500 male flies for each of actin-GAL4;EP(3)3247 (Hsp22+ flies) and actin-GAL4;TM3sb (control flies) were collected within 24h after eclosion in jars, and transferred into tubes (20 flies per tube) for determination of their differential survival rate during aging. The transferred 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. The flies were collected at the ages indicated (day 1, day 25, day 55 and day 93). For mitochondrial isolation, flies were used immediately without freezing.

4.3.2 Isolation of crude mitochondrial fraction from *Drosophila melanogaster*

Crude mitochondria were isolated from whole body of flies using slightly modified protocol, which was applied for mice mitochondria isolation by Melov et al., (1999) as described in Chapter 3. The procedure was optimized to use 200 flies for the isolation of crude mitochondria. Briefly, flies were placed on ice for 5 min, and then they were homogenized with 7ml of H-buffer (210mM mannitol, 70mM sucrose, 1mM EGTA, 5mM HEPES, pH 7.2) in 15ml of conical tissue grinder (Tissue Grinder System, VWR international, USA). Homogenization was done for 30-60 seconds until unified color of the extract was obtained. Subsequently, the homogenate was centrifuged at 1,000 x g, 4°C, for 5min. The supernatant was transferred to a new, cold 15ml of conical tube, and was re-centrifuged at 4°C, 1,000 x g for 5min. After removing floating debris such as broken wings from the obtained supernatant by passing a 100μm of nylon sieves (Cell Strainer REF 352360, BD Falcon, USA), a crude mitochondrial pellet was collected by centrifugation at 4°C, 8,800 x g for 10min and kept in -80°C until use. Following the procedure previously described in **2.3.3 SDS gel electrophoresis and immunoblotting**, pattern of Hsp22 expression in mitochondrial fraction during aging was examined, and compared with that in total homogenate fraction.

4.3.3 Experimental design for proteomic comparison

A comparative proteomic analysis can present various alterations in protein abundance resulting from different mechanisms. The over-expression of Hsp22 by GAL4/UAS system in *Drosophila melanogaster* showed a beneficial effect on prolonged lifespan. In addition to its influence on aging process, Hsp22 has a mitochondrial specific localization. Therefore, we performed a comparison specifically in the mitochondrial proteome between aged Hsp22+ flies (55-day-old) and its corresponding control to reveal the significant differential expression of mitochondrial proteins. For the corresponding control, a common pool of mitochondria that were obtained from Hsp22 over-expressing and normal-expressing flies at various ages (day 1, day 25 and day 55) was used. Thus, fly-individual or age-specific contributions could be normalized by comparison using this common pool of flies' mitochondria.

4.3.4 Comparative mitochondrial proteome analysis by high resolution IEF/ SDS-PAGE

Comparative proteomic analysis was performed with inverse replicate gels as described in Groebe et al. (2007), to obtain statistically significant results about differential protein abundance. Briefly explaining the process, a pair of samples, which were labeled with either ^{125}I or ^{131}I, were mixed at a concentration less than 1mg of protein, and separated by IEF/2D-PAGE high resolution covering a pH range of 4-9. For the same samples set, replicate gel separation was performed with reverse labeling to confirm their expressional reproducibility. A high sensitivity radio imaging technique was applied to discriminate between ^{125}I and ^{131}I signals in one 2D-PAGE gel and to generate a quantitative multicolor display of differential proteins from comparative samples labeled with different iodine isotopes. Statistical evaluation was based on at least four repeated data in each set of comparison. Gel image analysis was performed using the Pic/Greg software by the Fraunhofer Gesellschaft in Sankt Augustin as described in Hunzinger et al. (2006). Spot quantification and statistical identification of differential spots followed the description in Schrattenholz and Groebe (2007).

4.3.5 Protein identification by MALDI-TOF mass spectrometer

The selected protein spots were excised from the gel by a picking robot (ProPick, Genomic Solutions Ltd., Huntington, UK) and proteins in gel pieces were trypsin-digested using a ProGestrobot (Genomic Solutions Ltd., Huntington, UK). A ProMS-robot (Genomic Solutions Ltd., Huntington, UK) was used to apply samples for MALDI-TOF mass spectrometry onto an anchor target (Bruker, Bremen, Germany). Mass spectra of peptide ions were obtained using an Ultraflex MALDI time-of-flight (TOF) mass spectrometer in reflector mode within a mass range from m/z 800 to 4000. The MS spectra were calibrated and annotated automatically. The resulting peptide mass fingerprints were searched against the non-redundant NCBI Protein Sequence Database using Mascot Server software v. 1.8 (Matrix Science, London, UK).

4.3.6 Assay of complex I enzyme (NADH dehydrogenase) activity

The oxidation of NADH by the specific activity of complex I was measured at 340nm, 37℃ with adaptation to the methods shown in Trounce et al. (1996) and Barrientos (2002). The reaction mixture in a 1-ml quartz cuvette consisted of 250mM sucrose, 1mM EDTA, 50mM Tris-HCl, pH 7.4, 10μM decylubiquinone (DB), 2mM KCN, and approximately 50μg of mitochondrial protein. The reaction was initiated by adding 50μM NADH, and was monitored through the linear decrease of absorbance for 3min using a spectrophotometer (CARY BIO 100). Any inhibitor-insensitive activity by adding 5μg of rotenone was measured with the same procedure and was subtracted from the detected sample value. For this enzyme activity, the extinction coefficient ($5.5 mM^{-1} \cdot cm^{-1}$) was applied.

4.3.7 Assay of complex II enzyme (succinate dehydrogenase) activity

The reduction of 2,6-dichlorophenolindophenol (DCPIP) was monitored through the decreased absorbance at 600nm as shown in Trounce et al. (1996) and Barrientos (2002). Approximately 40μg of mitochondrial protein was incubated in a mixture of 50mM potassium phosphate, pH 7.4, and 20mM succinate at 30℃ for 10 min in 1ml cuvette. Next antimycin A (2μg/ml), rotenone (2μg/ml), KCN (2mM), and 50μM DCPIP were added and the blank rate was recorded for 1min. The reaction was initiated by adding 50μM DB, and the change in absorbance was monitored for 5min. For this enzyme activity, the extinction

coefficient applied was 19.1$mM^{-1} \cdot cm^{-1}$. The inhibitor-insensitive activity was determined by the same procedure with an addition of 10mM malonate into the incubation mixture.

4.3.8 Assay of complex III enzyme (cytochrome c oxidoreductase) activity

The reduction of cytochrome c was monitored through being catalyzed by complex III enzyme in the presence of reduced decylubiquinone (DBH_2) at 550nm (Trounce et al., 1996; Barrientos, 2002). The reaction mixture of 250mM sucrose, 1mM EDTA, 50mM Tris-HCl, pH 7.4, 50μM cytochrome c, 2mM KCN and 20μg of mitochondrial protein was incubated in a 1ml cuvette at 30℃ for 10min. The reaction was initiated by adding 50μM DBH2, and the increase in absorbance at 550nm was recorded for 1min. The inhibitor-insensitive activity was determined by the same procedure with an addition of 5μg/ml of antimycin A in the incubation mixture. A kinetic calculation was performed using first-order rate constants with extinction coefficient 19.0$mM^{-1} \cdot cm^{-1}$.

4.3.9 Assay of complex IV enzyme (cytochrome c oxidase) activity

Cytochrome c oxidase activity was measured by following the oxidation of reduced cytochrome c at 550nm (Trounce et al., 1996; Barrientos, 2002). For the preparation of reduced cytochrome c, first, 100mg of cytochrome c was dissolved in 1ml of 10mM potassium phosphate, pH 7.4. Then the cytochrome was reduced by adding 1ml of 0.1M sodium dithionite. The mixture was loaded onto a prewashed 20ml of Sephadex G-25 column, and the middle three fourths of the cytochrome band were collected. The full reduction of the cytochrome was checked by measuring the absorbance at 550 and 565nm, and the stock concentration was calculated from the absorbance at 550nm using an extinction coefficient of 27.7$mM^{-1} \cdot cm^{-1}$. The reduced cytochrome c stock was dispensed into 100μl aliquots under a gentle stream of nitrogen gas, and stored at -80℃.

For the measurement of complex IV activity, 20μM of the reduced cytochrome c was mixed with 10mM potassium phosphate (pH 7.4) in 1ml cuvette. The stability of the mixture was checked at 37℃ for 1min, and the reaction was begun by adding a 10μg of mitochondrial protein. The decreased absorbance at 550nm was quickly recorded for 2min. A kinetic calculation was performed using an extinction coefficient 19.0$mM^{-1} \cdot cm^{-1}$. The inhibitor-

insensitive activity was determined by the same procedure with an addition of 2.5mM lauryl maltoside (or 2.5mM KCN) in the incubation mixture.

4.3.10 Calculation of enzyme activities

For all above assays, the mitochondrial enzyme activities of each complex were calculated by following equation (Kramer et al., 2005).

$$\text{Enzyme activity / mg protein} = \frac{(\Delta\text{ABS/min})(\text{dilution factor})(1/\epsilon)(1000\mu\text{g/mg})}{(\mu\text{g/}\mu\text{l protein})}$$

Where ΔABS/min is the change in absorbance per minute; ϵ is extinction coefficient indicating a molar absorptivity (in $mM^{-1} \cdot cm^{-1}$). Assay procedure of mitochondrial complex activity was repeated for four or five times to confirm reproducibility and the obtained results were averaged for each final enzyme activity.

4.4 Results

4.4.1 Pattern of Hsp22 expression during aging

The pattern of Hsp22 expression during aging was examined in the whole fly body and the mitochondria. As shown in Fig. 4-1(A), the Hsp22 over-expressing flies showed developmental-stage specific variable expression of Hsp22. It appeared relatively abundant at the very young and old age. Comparatively, the control flies had an increased expression of Hsp22 only at the late stage of lifespan. In the mitochondrial fraction, the consistent pattern of Hsp22 expression was shown with highly concentrated Hsp22 especially at day 1 (Fig. 4-1(B)).

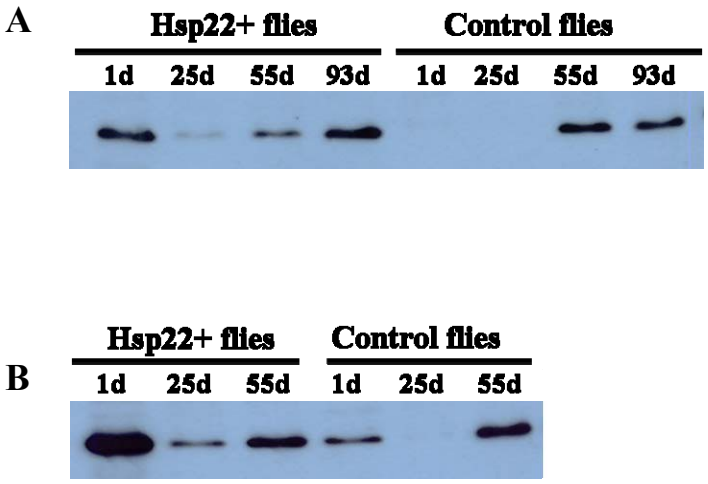

Fig. 4-1. The pattern of Hsp22 expression during aging of *Drosophila melanogaster.*
Equivalent amount of protein (20μg per lane) was applied for 12% SDS-PAGE and consequently Western immunoblot using antibody against Hsp22 (#36, 1/2500; Morrow et al., 2000). A) total homogenate fraction, B) mitochondrial fraction.

4.4.2 Long-lived Hsp22 over-expressing flies showed a differential profile in mitochondrial proteome

In the result of comparative mitochondrial proteomic analysis between Hsp22+ flies and common pool of control flies, inversely-repeated labeling of the compared mitochondrial proteins presented a reproducible alteration of spot abundance on 2D-gel separation (pI range 4-9) as shown in Fig. 4-2. In addition, the expression of proteins which are different from the normalized common pool of mitochondria can indicate a specificity shown in Hsp22 over-expressing flies. The 71 spots having different level of protein abundance were obtained from this comparison. Among those, 56 spots (79%) had a relatively higher level of abundance in the Hsp22+ flies and 15 spots (21%) showed a lower abundance. 25 spots among these were identified through MALDI-TOF mass analysis. 21 spots had higher abundance in the Hsp22+ flies as indicated in Table 4-1 and the other 4 spots were less abundant as shown in Table 4-2. Although most of these proteins have been indicated to localize in the mitochondria, some had unknown localization. They also included a cytoplasmic/cytoskeleton protein like Act87E.

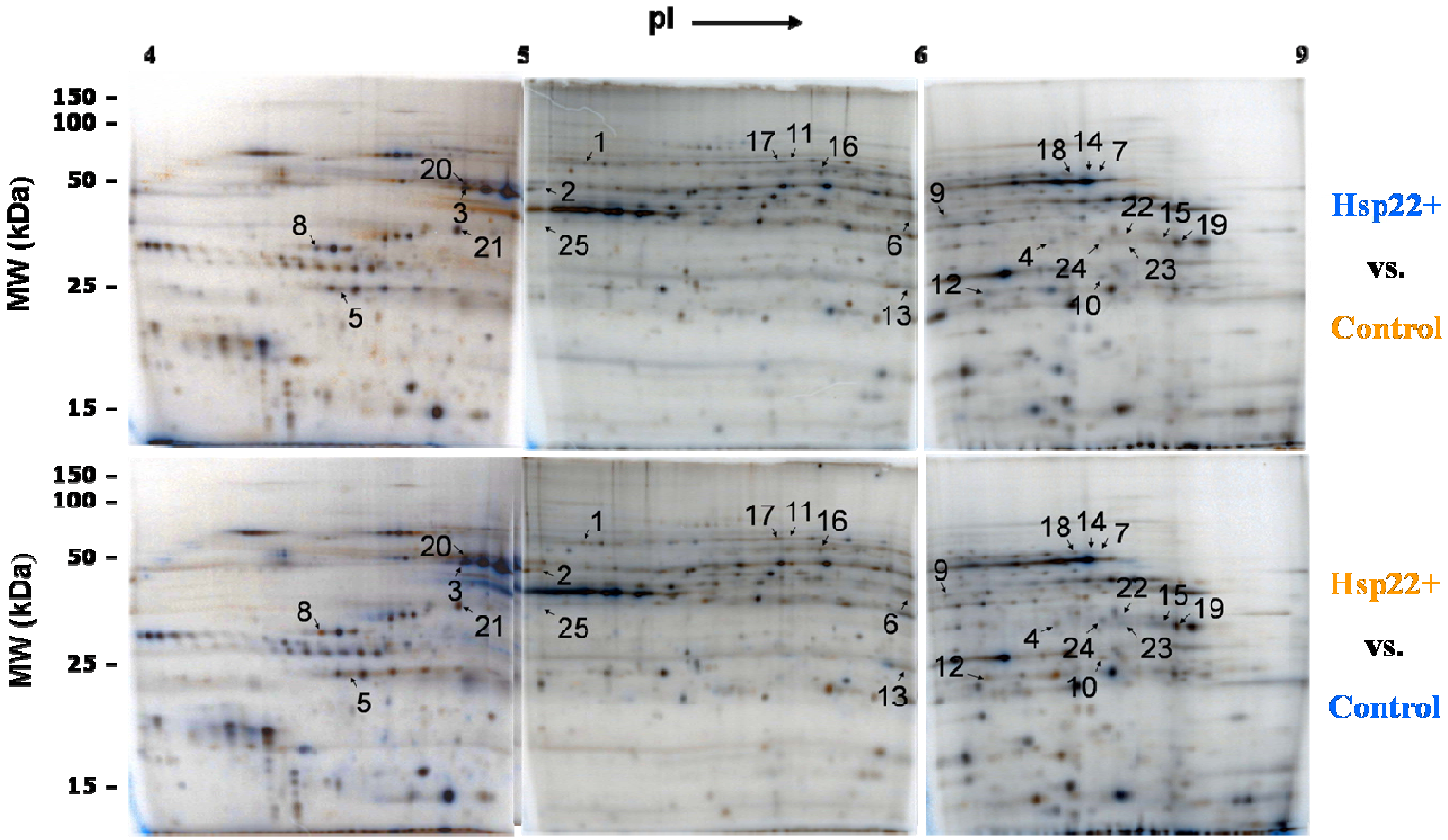

Fig. 4-2. Representative 2D-gel image of comparative mitochondrial proteome

In upper gels, Hsp22+ sample is labeled by ^{125}I (blue) and control sample is labeled by ^{131}I (orange). In bottom gels, the samples are convertly labeled. Spots showing differential abundance between Hsp22+ and control were directly excised from gels and identified by MALDI-TOF mass analysis (indicated as arrow mark).

Table 4-1. Proteins showing a higher level in mitochondria of Hsp22 over-expressing flies identified by MALDI-TOF mass analysis

Spot No.	Protein	Gene	Localization[a]	Ratio[b] (HSP/Control)	HSP[c] (%)	Control[d] (%)	p-value	Theoretical pI	Predicted MW(Da)	PMF[e] score	Accession Number NCBI
1	Vacuolar ATP synthase catalytic subunit A isoform 2	CG3762	Unknown (M)	1.28	56.2	43.8	0.0057	4.98	68303	125, 124, 107	gi|24583984, gi|19527547, gi|1373433
2	ATP synthase subunit beta, mitochondrial precursor	CG11154	M	1.31	56.7	43.3	0.0068	4.91 (or 10.21)	54109(or 13375)	359, 338, 177	gi|24638766, gi|287945, gi|34420370
3	ATP synthase subunit beta, mitochondrial precursor	CG11154	M	1.32	56.9	43.1	0.0001	4.91 (or 10.21)	54109(or 13375)	296, 295, 216	gi|287945, gi|24638766, gi|34420370
4	Probable 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor	CG15093	M	1.34	57.2	42.8	<0.0001	8.26 (or 11.10)	33883(or 24373)	144, 141	gi|19922568, gi|27820088
5	Mitochondrial glycoprotein, Q subcomponent binding protein	CG6459	MM	1.35	57.4	42.6	0.0010	4.73	28976	128, 113, 112	gi|20130085, gi|116806184, gi|116806188
6	Isocitrate dehydrogenase (oxidoreductase), TCA cycle	CG5028	M	1.43	58.9	41.1	<0.0001	6.8	44432	113	gi|24650122
7	Glutamate dehydrogenase, mitochondrial precursor	CG5320	MM	1.44	59.1	40.9	0.0071	8.58 (or 8.43)	61083(or 62537)	102, 91	gi|45553475, gi|542577, gi|45549226
8	Glutathione S-transferase S1	CG8938	Unknown	1.45	59.2	40.8	0.0005	4.29	27614	141	gi|24654347
9	Probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	CG12262	MM	1.47	59.5	40.5	<0.0001	8.09	45872	74	gi|24660351
10	Enoyl-CoA hydratase (oxidoreductation, fatty acid beta-oxidation)	CG6543	MM	1.51	60.1	39.9	<0.0001	8.85	31583	108	gi|20129971
11	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	CG2286	MIM	1.53	60.5	39.5	<0.0001	6.82	78631	259, 254, 190	gi|24640557, gi|40215555, gi|1808642
12	Alcohol dehydrogenase	CG3481	Unknown	1.54	60.7	39.3	<0.0001	7.57	27734	70	gi|38492426, gi|113424, gi|156800
13	Voltage-dependent anion-selective channel (porin, DmVDAC)	CG6647	MOM	1.55	60.8	39.2	<0.0001	6.97	30550	131	gi|1526607, gi|17136632
	NADH dehydrogenase (ubiquinone), mitochondrial respiratory chain complex I	CG12079	M	1.55	60.8	39.2	<0.0001	7.98	29971	124	gi|24656494

Table 4-1. (continued)

Spot No.	Protein	Gene	Localization[a]	Ratio[b] (HSP/Control)	HSP[c] (%)	Control[d] (%)	p-value	Theoretical pI	Predicted MW(Da)	PMF[e] score	Accession Number NCBI
14	Glutamate dehydrogenase, mitochondrial precursor	CG5320	MM	1.56	60.9	39.1	0.0015	8.58 (or 8.43)	61083(or 62537)	328, 293	gi|45553475, gi|542577, gi|45549226
15	L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	CG7998	MM	1.57	61.1	38.9	<0.0001	9.65	35318	252	gi|24647881
16	Malate dehydrogenase, TCA cycle	CG5889	M	1.57	61.2	38.8	<0.0001	6.69	68594	152, 150	gi|6634090, gi|21356279
17	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	CG2286	MIM	1.60	61.5	38.5	<0.0001	6.82	78631	283, 279, 229	gi|24640557, gi|40215555, gi|1808642
18	Glutamate dehydrogenase, mitochondrial precursor	CG5320	MM	1.62	61.8	38.2	<0.0001	8.58 (or 8.43)	61083(or 62537)	213, 212, 198	gi|542577, gi|45553475, gi|45549226
19	L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	CG7998	MM	1.69	62.8	37.2	<0.0001	9.65	35318	281	gi|24647881
20	Tubulin beta-2 chain	CG9359	Unknown	1.72	63.2	36.8	<0.0001	4.45	49871	106	gi|24645350
	ATP synthase subunit beta, mitochondrial precursor	CG11154	M	1.72	63.2	36.8	<0.0001	4.91 (or 10.21)	54109(or 13375)	97	gi|287945
21	Cathepsin D, proteolysis (eukaryotic aspartyl protease)	CG1548	Cyto	1.87	65.1	34.9	<0.0001	6.25	42473	153	gi|21355083

[a] subcellular localization of identified protein spot. M; Mitochondrion, MIM; Mitochondrion inner membrane, MOM; Mitochondrion outer membrane, MM; Mitochondrial matrix, Cyto; Cytoplasm (cytoskeleton)
[b] ratio means rate of protein amount in Hsp22+ mitochondria compared to control mitochondria
[c] relative abundance of Hsp22+ sample [= (Hsp22+) / (Hsp22+ plus Control) × 100%]
[d] relative abundance of control sample [= (Control) / (Hsp22+ plus Control) × 100%]
[e] peptide mass fingerprinting score

Table 4-2. Proteins showing a lower level in mitochondria of Hsp22 over-expressing flies identified by MALDI-TOF mass analysis

Spot No.	Protein	Gene	Localization[a]	Ratio[b] (HSP/Control)	HSP[c] (%)	Control[d] (%)	p-value	Theoretical pI	Predicted MW(Da)	PMF[e] score	Accession Number NCBI
22	L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	CG7998	MM	0.47	31.9	68.1	<0.0001	9.65	35318	197	gi|24647881
23	L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	CG7998	MM	0.66	39.7	60.3	<0.0001	9.65	35318	215	gi|24647881
24	L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	CG7998	MM	0.78	43.8	56.2	0.0001	9.65	35318	102	gi|24647881
25	Actin-87E	CG18290	Cyto	0.69	40.7	59.3	0.0008	5.15	41802	78	gi|21064361

[a] subcellular localization of identified protein spot. MM; Mitochondrial matrix, Cyto; Cytoplasm (cytoskeleton)
[b] ratio means rate of protein amount in Hsp22+ mitochondria compared to control mitochondria
[c] relative abundance of Hsp22+ sample [= (Hsp22+) / (Hsp22+ plus Control) × 100%]
[d] relative abundance of control sample [= (Control) / (Hsp22+ plus Control) × 100%]
[e] peptide mass fingerprinting score

4.4.3 Identification of proteins in the mitochondria displaying a higher abundance in Hsp22 over-expressing flies

After quantification of gel spots showing changes between the Hsp22 over-expressing flies and the control on the basis of their abundance ratio (HSP/Control), the proteins were picked and identified by MALDI-TOF mass analysis. As shown in Table 4-1, the identification of spots having a higher level of expression in Hsp22 over-expressing flies presented several functional proteins such as; ATP synthase subunit beta (1.31, 1.32, 1.72), mitochondrial glycoprotein (1.35), enoyl-CoA hydratase (1.51), glutamate dehydrogenase (1.44, 1.56, 1.62), isocitrate dehydrogenase (1.43), (L-)malate dehydrogenase (1.57, 1.69), NADH dehydrogenase (1.55), NADH-ubiquinone oxidoreductase 75kDa subunit (1.53, 1.60), probable 3-hydroxyisobutyrate dehydrogenase (1.34), probable medium-chain specific acyl-CoA dehydrogenase (1.47), and porin protein (1.55). These proteins are bona-fide mitochondrial proteins, and they are directly or indirectly involved in the process of energy production.

On the other hand, some of the spots having a higher level of abundance were proteins of unknown or uncertified subcellular location, such as alcohol dehydrogenase (1.54), cathepsin D protease (1.87), glutathione S-transferase S1 (1.45), tubulin beta-2 chain (1.72), and vacuolar ATP synthase catalytic subunit A isoform 2 (1.28). Among these, the most abundant protein in the flies over-expressing Hsp22 was ‘cathepsin D’ with the abundance ratio of 1.87 (65.1% of abundance in Hsp22 over-expressing flies versus 34.9% in common flies). This protein is one of aspartic-type (GAU, GAC) endopeptidases catalyzing hydrolysis of nonterminal peptide linkages in oligopeptides or polypeptides. Its optimum reaction pH is below 5 due to an aspartic residue involved in the catalytic process, and concomitantly this spot was detected on gel ranged in pI 4-5 as shown in Fig. 4-3.

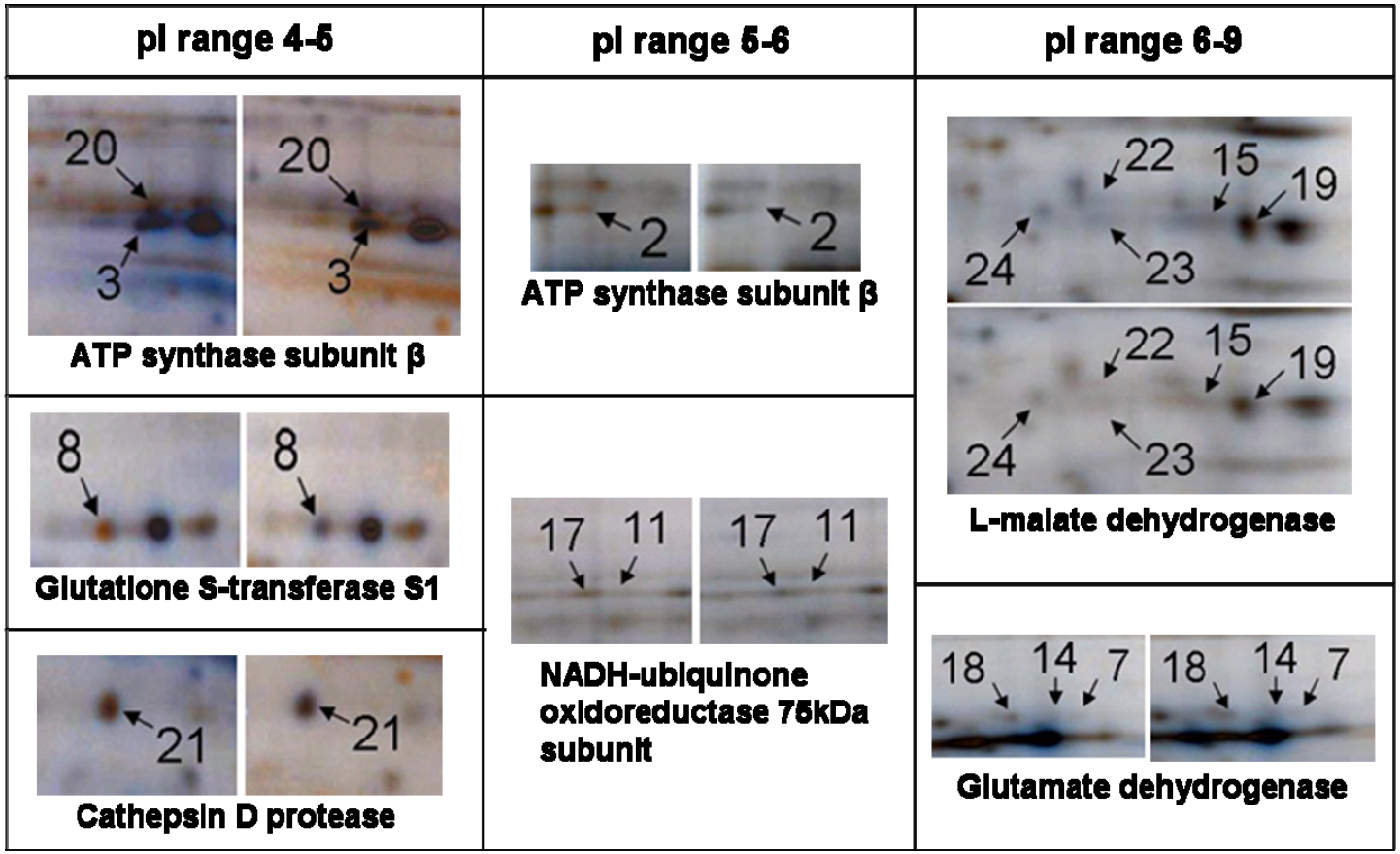

Fig. 4-3. Individual spot image showing reproducible abundance by dual color labeling

ATP synthase subunit β (3 spots), NADH-ubiquinone oxidoreductase 75kDa sununit (2 spots), L-malate dehydrogenase (5 spots), Glutamate dehydrogenase (3 spots) present several isoforms, or indicate possibility of posttlanslational modification. Spots of glutathione S-transferase S1 and cathepsin D protease clearly show the inversely labeled dual-color-image.

4.4.4 Identification of proteins showing lower abundance in Hsp22 over-expressing flies

Spots showing a lower level of expression in Hsp22 over-expressing flies were also identified (Table 4-2). Thus, the spots indicating homologous peptides to L-malate dehydrogenase were detected to have not only higher level of abundance as shown in section 4.4.2, but also lower level of abundance in Hsp22 over-expressing flies compared with common flies. The relative ratio of less abundant spots was a 0.5~0.7 fold with 30~40% of abundance in Hsp22 over-expressing flies vs. 60~70% in common flies ($p<0.0001$). A spot having a homology to Actin-87E also presented a lower level of abundance in Hsp22 over-expressing flies. Because this protein is known to be involved in cytoskeleton, the inclusion of Actin protein in a crude mitochondrial fraction can be a simple contamination from cytoplasmic fraction. Otherwise, its detection with the mitochondrial protein may result from its strong association with mitochondria. In any event, different expression of Actin may indicate a physiologically differential stage between long-lived Hsp22 over-expressing flies and common flies with correspondence to the prolonged lifespan.

4.4.5 Isoforms or possible posttranslational modification are detected in certain protein spots

The spot identification by MALDI-TOF presented certain proteins matching with the homologous sequence of peptides. From several spots at different molecular weight or different pI ranges, their identifications resulted in obtaining a single protein' name. This can be caused simply by protein isoforms, otherwise there is a possibility that certain proteins were modified in the posttranslational process. Moreover, the posttranslational modified proteins may have specific functional association.

As shown in Fig. 4-3, three different spots (spot 2, 3, and 20) with the homologous peptides of 'ATP synthase subunit beta' showed an increased expression in Hsp22 over-expressing flies relative to common flies. Spots 3 and 20 were displayed in a range of pI 4-5, whereas spot 2 was shown in a range of pI 5-6. A homology to 'NADH-ubiquinone oxidoreductase 75kDa subunit' was presented in the spot of 11 and 17 with pI range 5-6. Isoforms of 'Glutamate dehydrogenase' were identified from three different spots (spot 7, 14, and 18) in pI 6-9. On the other hand, spots homologous to 'L-malate dehydrogenase' were multiply detected in the range

of pI 6-9, such as spot 15, 19, 22, 23 and 24. Interestingly, two of these spots (spot 15 and 19) displayed higher abundance in Hsp22 over-expressing flies, whereas the rest showed a lower abundance (spot 22-24). This concurrent expression of either higher or lower abundance for the single protein 'L-malate dehydrogenase, implies the strong possibility of functional posttranslational modifications which may be relevant to the effect of Hsp22 on lifespan. Therefore, it can be hypothesized that alteration of abundance level of proteins involved in mitochondrial process such as TCA cycle, glycolysis and malate metabolism, is associated to longevity mechanism in flies over-expressing Hsp22.

4.4.6 The activities of mitochondrial respiratory chain complex enzymes during aging

Next, activities of the electron transport complexes in mitochondria were compared between Hsp22 over-expressing flies and control flies during aging (Fig. 4-4). The activity of complex I in Hsp22 over-expressing flies varied during aging and showed the highest activity at day 25 and the lowest activity at day 55, whereas the control flies had the highest activity at day 55. Hsp22 over-expressing flies had higher complex I activity than control flies at day 1 and 25, but not at day 55. In the case of complex II activity, Hsp22 over-expressing flies had lower activity than control flies at all ages, and the flies of day 1 had the highest activity. The activity of complex III was relatively larger than the activities of the other complexes. Moreover, the Hsp22 over-expressing flies showed predominantly higher complex III activity than control flies during aging. The pattern of complex III activity in both Hsp22 over-expressing flies and control flies showed higher activities at day 1 and 55 than day 25. Complex IV activity showed age-associated increase, and the activities between Hsp22 over-expressing flies and control flies were similar or a little higher activity in 55-day-old control flies.

There are variations in the pattern of age-associated changes in the activities of mitochondrial electron transport complexes between Hsp22 over-expressing flies and control flies. It seems that there is no single uniform pattern in activities of mitochondrial electron transport complexes during aging. However, the majority of complex activities were relatively higher in Hsp22 over-expressing flies than in control flies except for the cases of complex II and 55-day-old complex I and IV.

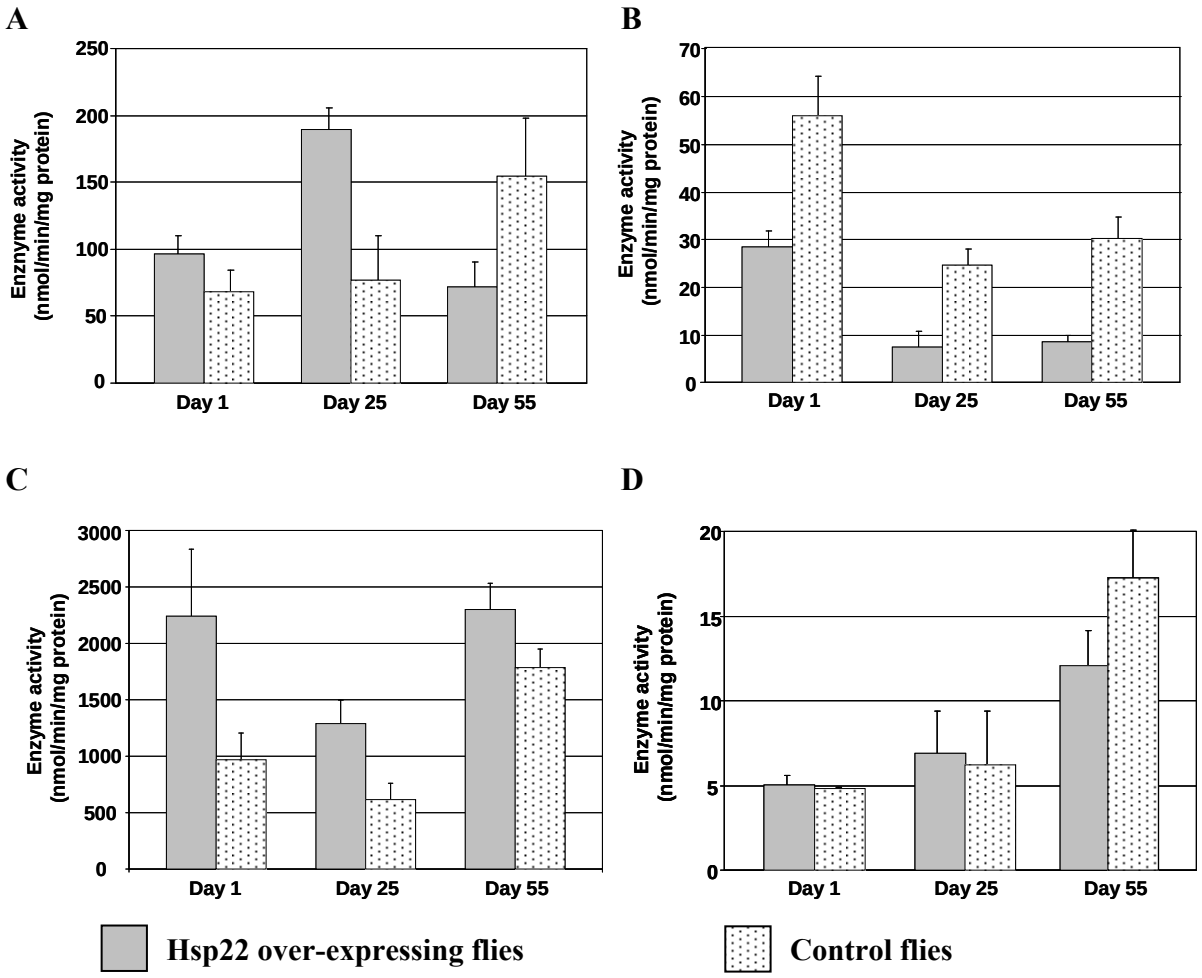

Fig. 4-4. Activities of mitochondrial electron transport complexes in Hsp22 over-expressing and control flies

Crude mitochondrial proteins isolated from Hsp22 over-expressing flies and control flies at day 1, day 25 and day 55 were utilized for the assay of activities of mitochondrial electron transport complexes. The assay procedure was repeated for four or five times to confirm reproducibility and the final enzyme activity was obtained by averaging them. A) complex I enzyme (NADH dehydrogenase) activity, B) complex II enzyme (succinate dehydrogenase) activity, C) complex III enzyme (cytochrome c oxidoreductase) activity, D) complex IV enzyme (cytochrome c oxidase) activity.

4.5 Discussion

From the overall data of comparative mitochondrial proteomes, proteins showing differential expression in the long-lived Hsp22 over-expressing flies suggest key functions potentially involved in Hsp22-associated longevity mechanism. In this study, most of the proteins displaying differential levels of abundance are involved in the functions of mitochondrial respiratory complexes, TCA cycle, fatty acid beta oxidation, voltage-dependent anion-selective channel, amino acid metabolism, and anti-oxidative response.

The relative over-expression of 'ATP synthase subunit beta' was observed in human colonic epithelium tissues of young people compared with that of old people (Li et al., 2007). Redundant spots of ATP synthase subunit isoforms, showing age-associated differential expression were also observed in the mitochondria of various organisms (rat brain, P. anserina, HUVEC) (Groebe et al., 2007). This finding suggests that functional inactivation of ATP synthase could be induced by ROS-mediated posttranslational modification as shown in its various spots altered during aging. During *Drosophila* aging, a continuous decline of transcripts of 'ATP synthase subunit beta' encoded in the nuclear genome was also observed concomitantly with decline of 16SrRNA, cytochrome b and cytochrome c oxidase subunit I encoded in the mitochondrial genome (Calleja et al., 1993). This is a consistent with the increased protein expression of 'ATP synthase subunit beta' observed in long-lived Hsp22 over-expressing flies. Interestingly, Cermelli et al. (2006) identified this mitochondrial protein from the lipid-droplet fraction with an early *Drosophila* embryonic histone enrichment, suggesting the possibility of a dynamic exchange of proteins between lipid droplets and mitochondria. As protein of similar function, 'vacuolar ATP synthase catalytic subunit A isoform 2' was one of more abundant proteins in the flies over-expressing Hsp22. Increase of these proteins involved in the ATP synthase subunits suggests a role of Hsp22 effectively maintaining the energy metabolism.

From the result of Cermelli et al. (2006), 'L-malate dehydrogenase' was also involved in the proteome of lipid droplets as a mitochondrial protein. Because spots of higher abundance as well as lower abundance for this single protein were concurrently detected, it might imply that

posttranslational modifications are also related to a role of Hsp22 in prolonging lifespan. Main function of this enzyme is to oxidize L-malate into oxaloacetate producing NADH in TCA cycle. Therefore, the expression of 'L-malate dehydrogenase' may indicate specific reactions for TCA cycle within mitochondrial matrix of Hsp22 over-expressing flies. Furthermore, the concomitant increase of 'isocitrate dehydrogenase' supports the possibility that Hsp22 has an effect on TCA cycle. The increase of proteins constituting mitochondrial respiratory chain complex I such as 'NADH-ubiquinone oxidoreductase 75 kDa subunit' and 'NADH dehydrogenase', is another clue for the effect of Hsp22 in mitochondrial function (oxidative phosphorylation pathway).

On the other hand, the relative abundance of proteins such as 'Acyl-CoA dehydrogenase' and 'Enoyl-CoA hydratase' indicates the functional involvement in fatty acid beta-oxidation pathway. Moreover, these two enzymes are also linked to amino acid metabolism (valine, leucine and isoleucine degradation, beta-Alanine metabolism) and carbohydrate metabolism (propanoate metabolism). The abundant spot having homology to '3-hydroxyisobutyrate dehydrogenase' shows additional possibility of amino acid metabolism involvement (valine, leucine and isoleucine degradation). Interestingly, this protein has been shown to be included in lipid droplet fraction as a mitochondrial protein (Cermelli et al., 2006). Taken together, the common function of these three proteins indicates the possibility that Hsp22 might be involved in the amino acid degradation process, especially for valine, leucine and isoleucine.

Porin, a major protein of the outer mitochondrial membrane in eukaryotes is well known as the 'voltage-dependent anion-selective channel (VDAC) activity' (Dihanich, 1990; Mannella, 1992; Benz, 1994). Specifically, it forms a channel through the membrane that allows diffusion of small hydrophilic molecules. In this study, *Drosophila* 'porin' protein (DmVDAC) was shown to be relatively abundant in Hsp22 over-expressing flies, and might indicate a possibility of contribution to longevity. Consistent with this result, Kang et al. (2002) reported that feeding PBA (4-phenylbutyrate) caused a lifespan extension in *Drosophila* with increasing transcriptional level of the DmVDAC gene. Furthermore, the putative association between mitochondrial DmVDAC protein and lipid droplet was reported by Cermelli et al. (2006). Another mitochondrial protein, 'glycoprotein-like CG6459-PA' which is involved in defense response to bacteria was abundant in Hsp22 over-expressing flies, and this may elicit beneficial effects on longevity.

An increased abundance of ‘glutamate dehydrogenase’ located in the mitochondrial matrix was detected in three different homologous spots. During amino acid biosynthesis, this enzyme catalyzes the reductive amination of α-ketoglutarate to glutamate. Surplus amino acids of glutamate precursor cause their degradation, and are reused as metabolic fuel. Therefore, the activity of ‘glutamate dehydrogenase’ is allosterically regulated. Specifically, guanosine triphosphate (GTP) and adenosine triphosphate (ATP) are allosteric inhibitors, whereas guanosine diphosphate (GDP) and adenosine diphosphate (ADP) are allosteric activators (Stryer, 1995).

In addition, the abundance of ‘Glutathione S-transferase S1’ in Hsp22 over-expressing flies is consistent with beneficial effect of the anti-oxidative response on longevity. Generally, glutathione S-transferases (GSTs) have been known to catalyze the conjugation of glutathione (GSH) with variable electrophilic compounds and to play a major role in detoxification processes (Habig et al., 1974; Ketterer et al., 1976). Although the exact subcellular localization of GSTs is still unknown, there are several reports mentioning the possibility that GSTs can localize to mitochondria, where they may play a role in modulating the consequences of ROS production (Bhagwat et al., 1998; Huang and Philbert, 1995). Interestingly, disruption of a microsomal GST-like gene appeared to reduce lifespan in *Drosophila melanogaste*r, which is possibly caused by decreased efficacy in detoxifying lipid peroxidation, particularly in the membrane compartment of cells (Toba and Aigaki, 2000). GSTs have been shown not only to have a transferase function, but also to serve a regulatory role in the balance between cell survival and apoptosis through protein-protein interactions with members of the mitogen activated protein (MAP) kinase pathway (Adler et al., 1999; Yin et al., 2000; Cho et al., 2001). Thus, the abundant expression of GST in flies over-expressing Hsp22 can also provide another plausible explanation for the mechanism of extended lifespan.

The presence in mitochondrial fraction of several proteins having unknown localization may present a possibility of additional new mitochondrial proteins, or may indicate a close association with mitochondria. One of these, ‘alcohol dehydrogenase’ plays an essential role in both the detoxification of acetaldehyde by transforming acetaldehyde into ethanol and the metabolic use of ethanol in *Drosophila melanogaster* (Leal and barbancho, 1992). ‘Tubulin beta-2 chain’ is a structural constituent of the microtubular cytoskeleton, and is involved in GTP binding as a highly conserved biochemical and molecular structure varying from

unicellular organisms to man (Sanchez et al., 1980; Raff, 1984, Goldstein and Gunawardena, 2000). Interestingly, Hsp22 over-expressing flies had abundant levels of 'tubulin beta-2 chain', while they showed a decreased level of 'Act87E'. The relationship between cytoskeleton structure and the aging process, as well as their presence in the mitochondrial fraction remains unexplained at this time. Although Labuhn and Brack (1997) showed age-dependent or tissue-specific decrease of mRNA of Act87E gene, the multiple isoforms of Actin might be involved in various cellular functions such as cytoskeleton structural cell mobility, chromosome movement and muscle contraction.

More interestingly, 'Cathepsin D' which is a ubiquitous aspartic protease showed the most striking change in abundance in Hsp22 over-expressing flies. This enzyme is involved in autophagic salivary gland cell death during *Drosophila* metamorphosis (Gorski et al., 2003), and flies mutant for this gene exhibit key features of neuronal ceroid lipofuscinoses (NCLs) and an age-dependent neurodegeneration (Myllykangas et al., 2005). Moreover, the enzyme has been associated with various biological processes such as apoptosis (the release of cytochrome c from mitochondria) (Roberg et al., 1999), the loss of the transmembrane potential, aging, Alzheimer's disease, and breast cancer. Therefore, the function of proteolysis may be a very plausible factor involved in lifespan extension in Hsp22 over-expressing flies.

There are several reports indicating age-related decline of mitochondrial chain complexes activities. Miquel et al. (1995) reported an approximately 30% loss of complex I, II+III, and IV activity with age in mouse liver mitochondria. According to Desai et al. (1996), substantial age-related declines occur in the activities of complex I, III, and IV in the mouse gastrocnemius muscle. Others have shown an age-associated decline in the activities of complex I and IV in human skeletal muscles (Cooper et al., 1992). Nevertheless, whether or not activities of mitochondrial electron transport complexes uniformly decline during aging still remains controversial. For instance, Barrientos et al. (1996) found no correlation between specific activities of electron transport complexes and age. The pattern of age-related changes in the enzymatic activity of the complexes observed in liver mitochondria, was dissimilar from that observed in mitochondria isolated from skeletal muscle, heart, and brain (Guerrieri et al., 1993; Bowling et al., 1993; Ferrándiz et al., 1994; Desai et al., 1996).

The most noticeable age-related decline in the present study, however, was the reduced activity in complex II. On the contrary, an age-related increase of complex activity was detected in complex IV. Activity of complex I appeared to peak at day 25 and decline at day 55 in the Hsp22 over-expressing flies, while it showed an age-associated increase in control flies. The age-related pattern in the activities of complex III declined during the first part of life and increased during the latter part. Taken together, the overall pattern of the activity of the respiratory chain complexes in both Hsp22 over-expressing flies and control flies was quite variable and not simply age-associated decline. Although the results observed in our study do not confirm the age-associated decline in their activity as reported previously in mouse (Miquel et al., 1995), there are certain representative patterns among the activities. For example, the Hsp22 over-expressing flies consistently have higher activities in complex I and IV, at the early (day 1) and mid (day 25) age, whereas lower activities at old (day 55) age than control flies. Complex II activities are higher in control flies, whereas complex III activities are predominantly higher in Hsp22 over-expressing flies.

Variable pattern of the respiratory chain complexes activities shows specificity depending on type of tissues or other possible effectors (Kwong and Sohal, 2000). For example, aging process could have an effect on the enzyme protein mass or catalytic potential of the coupled complexes. Other possibilities are phospholipid membrane environment surrounding the protein complexes or alterations in the mitochondrial membrane fluidity. Besides, the activity of cytochrome c oxidase (complex IV) has been shown to be controlled allosterically by the intramitochondrial ATP/ADP ratio (Gnaiger et al., 1998). Therefore, on the regulation of respiratory chain activity, there seem to be additionally considerable impacts as well as a generation of proton gradient.

Higher activities in parts of mitochondrial electron transport complexes in Hsp22 over-expressing flies is consistent with the result of increased expression of proteins involved in mitochondrial energy metabolism. In addition, Hsp22 over-expressing flies have remarkably higher activities of complex III regardless of ages than control flies. Interestingly, the complex III is considered to be crucial for the activity of the entire respiratory chain because the unit value of the activity in complex III was the highest among the other complexes, indicating their important participation in ROS production.

In summary, Hsp22 over-expressing flies presented relatively higher abundance of proteins such as mitochondrial respiratory complexes, TCA cycle, fatty acid beta oxidation, voltage-dependent anion-selective channel, amino acid metabolism, anti-oxidative response, and proteolysis. Most notably, the ubiquitous aspartic protease, Cathepsin D, was relatively the most abundant protein in Hsp22 over-expressing flies. The differential expression of specific enzymes in long-lived Hsp22 over-expressing flies may suggest some mechanisms in the extension of lifespan.

4.6 Acknowledgements

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Chapter 5. Transcriptional profiling of aging in flies over-expressing the small mitochondrial chaperone Hsp22

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5.1 Abstract

Aging is a complex process, which is accompanied by a decreased capacity to tolerate and respond to various forms of stresses. Heat shock proteins (Hsps) are part of the cell defense mechanism and are up-regulated by stress. In *Drosophila melanogaster*, the small heat shock protein (sHsp) Hsp22 is localized in the mitochondrial matrix and is preferentially up-regulated during aging. Its over-expression resulted in an extension of lifespan and an increased stress resistance (Morrow et al., FASEB J. 18, 598, 2004). Hsp22 has chaperone-like activity *in vitro*, but the mechanism by which it increases lifespan in flies is still unknown.

Ubiquitous over-expression of Hsp22 in *Drosophila* using the binary GAL4/UAS system resulted in a ≈30% increase in mean lifespan. We have performed a genome-wide analysis on long-lived Hsp22 over-expressing and normal-lived control flies to unveil transcriptional changes brought by Hsp22. We have compared transcriptomes according to the fly chronological and physiological ages. Flies over-expressing Hsp22 display an up-regulation of genes normally down-regulated with age. These are involved in mitochondrial function, protein biosynthesis, proteolysis and defense response. Genomic analysis suggests that Hsp22 plays a role in lifespan determination by altering the regulation of genes involved in the aging process.

5.2 Introduction

Aging is a complex phenomenon. It is accompanied by a decreased capacity to tolerate and respond to various forms of stress, and consequently it has been associated with many diseases. Many theories have been postulated to explain the aging process (reviewed in Viña et al., 2007); among them, the free radical theory of aging of Harman (1956) has gathered large support but is still debated (Sanz et al., 2006; Muller et al., 2007). According to this theory, aging is mainly caused by the accumulation of macromolecular damages induced by toxic reactive oxygen species (ROS). Since mitochondria are the main generators of ROS as a by-product of ATP synthesis (Aguilaniu et al., 2005), they are at the center of the free radical theory of aging (Harman, 1972; Miquel et al., 1980; reviewed in Viña et al., 2007).

Genome-wide experiments in different model organisms have identified shared transcriptional profile in aging across species (McCarroll et al., 2004; Smith et al., 2007). Mitochondrial genes including many components of the mitochondrial respiratory chain, ATP synthase complex and citric acid cycle are repressed during aging as well as genes involved in ATP-dependent transport such as primary active transporters, ions transporters and ABC transporters (McCarroll et al., 2004). Interestingly, the repression of these genes is implemented early in adulthood (McCarroll et al., 2004).

Several mutants displaying increased longevity in *Caenorhabditis elegans* and *Drosophila melanogaster* have been described. Interestingly in addition to having an extended longevity, many of them displayed increased thermotolerance and resistance to stress (Lin et al., 1998; Rogina et al., 2000; Chavous et al., 2001; Kitagawa et al., 2000; Nakamoto et al., 2000). Heat shock proteins (Hsps) are molecular chaperones that are coordinately expressed in response to stress and have been shown to be major determinants in the acquisition of thermotolerance and stress resistance (Feder et al., 1999; Verbeke et al., 2001). Along with the antioxidant defense system and the ubiquitin/proteasome machinery, Hsps are part of the cell-defense mechanisms to prevent accumulation of protein damages (Ehrnsperger et al., 2000; Hartl and Hayer-Hartl, 2002; Haslbeck, 2002; Walter and Buchner, 2002).

Hsps are divided in four subfamilies on the basis of their molecular weight and sequence homology; small Hsps (sHsps), Hsp60, Hsp70 and Hsp90/100. In *Drosophila melanogaster* there are four main sHsps displaying distinct intracellular localization and developmental expression pattern (reviewed in Michaud et al., 2002). Hsp22 is localized in the mitochondrial matrix (Morrow et al., 2000) and has been shown to be preferentially up-regulated in aging flies (King and Tower, 1999). Interestingly, flies selected for their increased longevity display an earlier onset of *hsp22* and *hsp23* transcription (Kurapati et al., 2000). Recently, we have shown that over-expressing Hsp22 in motoneurons is sufficient to increase lifespan and resistance to oxidative stress by more than 30% (Morrow et al., 2004a). Most importantly, long-lived flies over-expressing Hsp22 maintain their fitness for a longer period as revealed by negative geotaxis (Morrow et al., 2004a). Arguing for a beneficial role of Hsp22 during aging, flies carrying a P-element preventing normal Hsp22 expression have a reduced lifespan and resistance to stress (Morrow et al., 2004b). *In vitro*, Hsp22 has a chaperone-like activity as many model substrates (Morrow et al., 2006; Heikkila et al., 2007), but its function *in vivo* is still undetermined.

We have performed a genome-wide transcriptional analysis on long-lived Hsp22 over-expressing flies and normal-lived control flies to unveil the transcriptional changes related to Hsp22 over expression and have insights on how Hsp22 exert its beneficial effect on aging in *Drosophila*. Transcriptomic profiles were compared according to the chronological and physiological ages of flies. From the analysis of microarray data, Hsp22 over-expression results in mostly up-regulations rather than down-regulation of genes. Flies over-expressing Hsp22 display an up-regulation of genes normally down-regulated with age. These genes are involved in mitochondrial function, protein biosynthesis, proteolysis and defense response.

5.3 Materials and Methods

5.3.1 Fly strains, maintenance and longevity

The *actin*-Gal4 strain (Flybase ID: FBti0012293) contains 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, Rørth, 1996) contains a UAS-including P-element inserted 643bp upstream of the *hsp22* translation initiation codon (FlyBase Genome Annotators, 2002-2003) on the third chromosome, and through an activation of the UAS by Gal4 results in Hsp22 over-expression (Figure 1) (Brand and Perrimon, 1993). Males of siblings obtained from the crossing between *actin*-Gal4/*cyo* males and EP(3)3247/TM3,*sb* females were used in the microarray experiments. In analysis, *actin*-Gal4;EP(3)3247 flies over-expressing Hsp22 (Hsp22+) were used as a target sample and compared with two different controls of *cyo*;EP(3)3247 (control 1) and *actin*-Gal4;TM3,*sb* (control 2) in parallel at corresponding ages.

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, 1.8% (v/v) tegosept. For the longevity experiments, male flies (Hsp22+, control 1 and 2) were collected within 24 hours after hatching and put into tubes (20 flies per tube). Flies were put on fresh food and scored for survivorship every 3-4 days. Flies were collected at the different chronological ages (day 1 and day 45) or different physiological ages (corresponding to 95%, 90% and 50% survival). Two or three fly cohorts of each age conditions were collected. After collection, flies were kept at -80°C until RNA extraction.

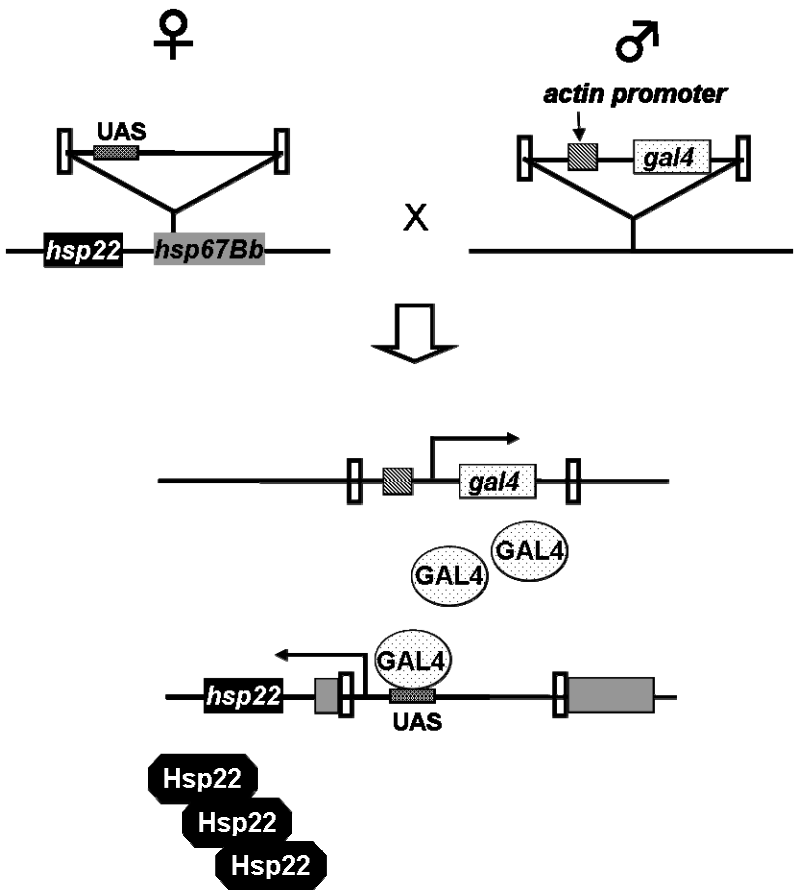

Fig. 5-1. Hsp22 over-expression by GAL4/UAS activation

The EP(3)3247 strain contains a UAS-including P-element insertion at 643bp upstream of the *hsp22* translation initiation codon, and through an activation of the UAS by *actin*-promoted Gal4, results in Hsp22 over expression.

5.3.2 Microarray chip used

Array chips were supplied by CDMC (Canadian *Drosophila* Microarray Centre, http://www.flyarrays.com/). *Drosophila* 12K version 1 array contains 25,640 spots corresponding to 10,500 genes and some control spots. The list of spotting clones was collected from several different sources: Berkeley DGC 1.0 UniGene EST set (5900 clones), B. Oliver's and J. Andres's non-DGC EST clones (1078 clones), genes from submitted gene lists (572 genes), various control genes (LacZ, GFP, Gal4, genes to calibrate RNA amounts, tetracycline transactivator, TAP tags, pBS, pOT), and 5530 Berkeley *Drosophila* Genome Project DGC 2.0 clones. Further details on chip production can be found in Neal et al. (2003).

5.3.3 Total RNA extraction, cDNA labeling and microarray chip hybridization

Flies were homogenized with a disposable Tissue Grinder System (VWR International) and total RNA was extracted with Trizol (Invitrogen, Gibco-BRL) as recommended by the manufacturer. To remove any genomic DNA contamination, total RNA was purified with the MEGAclearTM Kit (Ambion). RNA preparations for Hsp22+ and controls were done by pair, and equal amounts of RNA (60 ~ 80 μg) were used as templates for reverse transcription according to the manufacturer (Invitrogen life Technologies). Hsp22+ samples were labeled with fluorescent dye Cy5-dCTP (NEN) and control samples were labeled with Cy3-dCTP (NEN). Hybridization of cDNA probes on microarray chips was performed at 37 °C during 12-18 hours.

5.3.4 Microarray data processing and analysis

Cy5 and Cy3 fluorescence intensities were detected using ScanArray 4000 system (GSI Lumonics/Perkin Elmer). Scanned 16-bit Tiff images were quantified by GenePix Pro 6.0 software (Axon Instruments). Further procedures for data normalization and filtration were processed by MultiExperiment Viewer Version 4.0 beta. Raw data were normalized by the iterative linear regression method as the global mode with ± 2.0 SD range. The spots' intensities were filtered with a bottom line of average background intensity for each of Cy5 and Cy3. In T-test and 2-way ANOVA analysis, p-values below 0.01 were accepted.

As mentioned above, total RNA was extracted from two or three fly cohorts for each age condition and each control. Therefore, for a determined age, two or three different arrays were hybridized per control. For a given control, genes showing reproducible expression for at least 4 repeated spots (one gene corresponds generally to two spots on one chip) were considered. To remove the effect of genetic background, genes showing a common expression with both controls (8 to 12 repeated spots) were further analyzed. The functional analysis about the genes showing changed expression was performed according to the classification by Flybase Gene Ontology database (http://bugbane.bio.indiana.edu:8092/). A similarity test by genes clustering was executed to identify genes showing a synchronized age-dependent pattern of transcription with *hsp22* gene. In this, Hierarchical clustering (HCL) was optimized with the average-linkage method of 'Euclidean Distance Metrics' (Eisen et al., 1998) to visualize the closely-linked genes having similar transcriptional profile with *hsp22*.

5.3.5 RT-PCR and gel analysis

Transcript levels of some selected genes were measured using RT-PCR in order to validate microarray data. The primer design for testing these genes was carried out using DNAClub software, and primer pairs were selected to span an intron/exon junction, except for a few genes without intron. The designed primer sets were purchased from Integrated DNA Technologies, Inc. (Coralville, IA). Total RNA samples were DNase-treated with TURBO DNA-*free*™ (Ambion, TX, USA). First-strand of cDNA was synthesized by a reverse transcription from the RNA sample using RETROscript Kit (Ambion, TX, USA) following the manufacturer's instruction. Then, 5µl of the obtained RT reaction was combined with 5µl of 10 × PCR buffer, 2.5µl of 10mM dNTP mix, 2.5µl of PCR primers (mixture with 5 µM of forward and reverse primers: *gal4* primer set of 5'-GCAGCATTCTGGAACAAAGACG-3', 5'-GTAGGGTATTGGGCGATAGTTGC-3'; *hsp22* primer set of 5'-ACTATCATGAGGTCCTTACCG-3', 5'-TAACTCGAGACTTATTTCTACTGACTGGC-3'), 34.5µl of nuclease-free water and 0.5µl of *Taq* DNA polymerase (GE Healthcare, UK) in 50µl of total volume for PCR reaction. The combined solution was subjected to an initial denaturation at 94°C for 2min in thermal cycling machine (Flexigene, Techne Inc., Cambridge, NJ, USA), and then 35 cycles at 94°C for 30sec, 55°C for 30sec, and 72°C for 1 min. At the end of the final cycle, an additional extension step was performed for 5min at

72°C. After PCR procedure, the obtained reaction was mixed with 6 × loading buffer, loaded into 2% agarose gel (Invitrogen life technology). Electrophoresis was performed at 100V for 45min. The migrated gel was stained in TAE buffer containing ethidium bromide (1mg per 100ml) in a dark container. The gel image was visualized and quantified using the Chemi Genius2 imaging system and Genesnap image acquisition software (Syngene, Ontario, Canada).

5.4 Results

5.4.1 Hsp22 over-expression prolongs flies lifespan

To perform our microarray experiments we have chosen to drive Hsp22 over-expression ubiquitously using an *actin* promoter (*actin*-Gal4) as its effects on aging are similar to the motorneurons targeted (D42-Gal4) over-expression (Morrow et al. 2004a) and it allows usage of whole flies to isolate RNA. Hsp22 over-expression was achieved by using the GAL4/UAS system (Fig. 5-1, Additional data file A) and male siblings of *actin5C*-Gal4;EP(3)3247 (Hsp22+) flies were used as control to avoid interference from multiple fly genetic backgrounds.

As shown in Fig. 5-2, Hsp22+ flies have a longer lifespan than both controls (*cyo*;EP(3)3247 (control 1) and *actin*-Gal4;TM3,*sb* (control 2)). At 50% survival, Hsp22+ flies were ≈ 65days old while control 1 and 2 flies were ≈ 46days old, which correspond to a 29.2% increase in mean lifespan. The maximum age of Hsp22+ flies was ≈110 days compared to ≈ 90days in controls. This result is consistent with previous studies on the effect of Hsp22 over-expression on longevity (Morrow et al., 2004a). Based on these longevity curves, several sampling points were chosen to carry out a comparative genome-wide analysis. Flies were collected at two chronological ages (day 1 and day 45) and three physiological ages (95%, 90% and 50% survival). Chronological and physiological ages have also both been used in another microarray study on MnSOD-mediated extension of lifespan in *Drosophila* (Curtis et al., 2007). If we assume that Hsp22 simply extends lifespan and the normal time course of gene expression, differences observed at the same chronological age should include both targets of Hsp22 and potential biomarkers of aging that have their time course delayed. On the other hand, differences observed at the same physiological age should include both targets of Hsp22 and alterations that do not simply represent a delay in normal aging patterns. Moreover, genes whose expression is commonly altered in both the 45-day-old data set (same chronological) and 50% survival data set (physiological age) should represent primary targets of Hsp22 (Fig. 5-3).

We used two different controls in the microarray experiments, each one containing either ‘EP(3)3247’ or ‘*actin*-Gal4’, to eliminate the effects of genetic background and P-element

insertion. Therefore, genes showing similar changes in comparison with both control 1 and 2 were further analyzed.

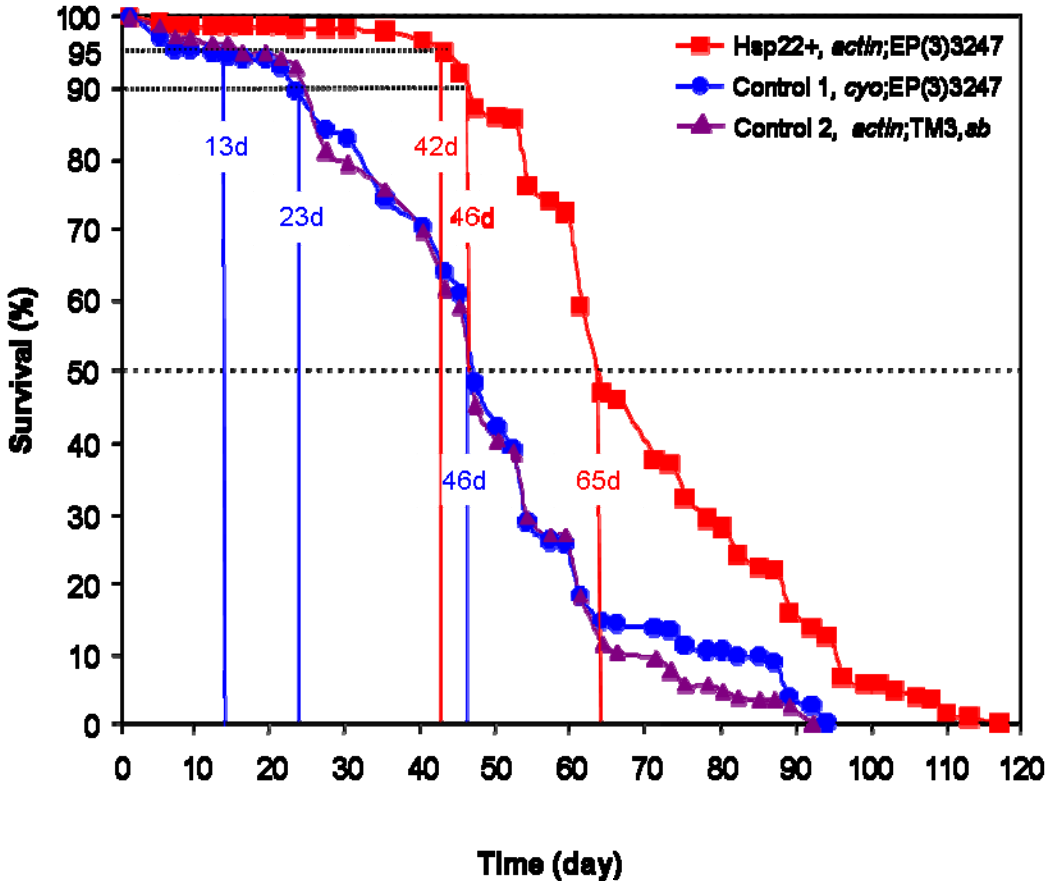

Fig. 5-2. Lifespan extension by Hsp22 over-expression

0-24h *actin*-Gal4;EP(3)3247 (Hsp22+), *cyo*;EP(3)3247 (control 1) and *actin*-Gal4;TM3,*sb* (control 2) male flies were collected and scored for survival every 3-4 days. For the comparative genome-wide analysis, fly samples were collected at different chronological (day 1 and day 45) and physiological ages (95%, 90% and 50% survival).

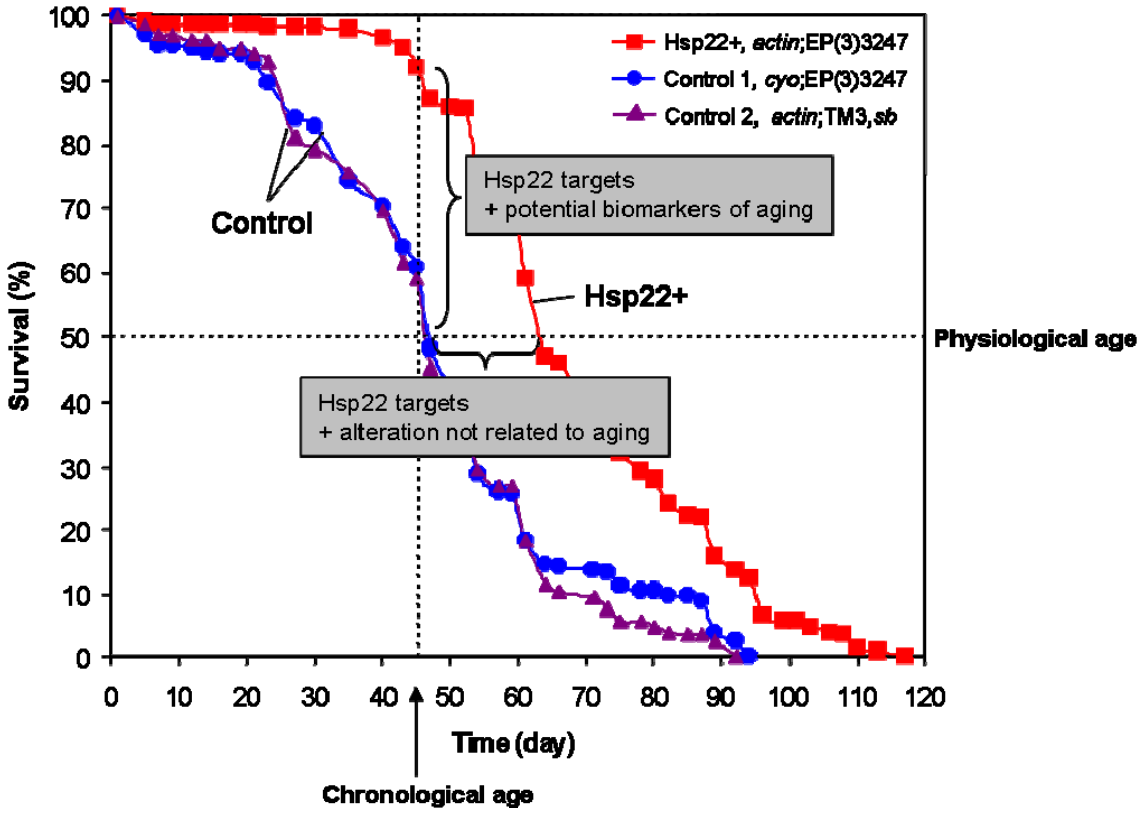

Fig. 5-3. Information brought by the 45 days-old and 50% survival flies data sets

This figure is based on Figure 2. Assuming that Hsp22 simply extends lifespan and the normal time course of gene expression, differences observed at the same chronological age should include both targets of Hsp22 and potential biomarkers of aging that have their time course delayed. On the other hand, differences observed at the same physiological age should include both targets of Hsp22 and alterations that do not simply represent a delay in normal aging patterns. Therefore, genes whose expression is altered in the same direction for both the 45 days-old data set (same chronological) and 50% survival data set (physiological age) should represent primary targets of Hsp22.

5.4.2 Genes are mainly up-regulated in Hsp22 over-expressing flies

Genome-wide transcriptional profiles were processed by combining data from both controls under each single age conditions. Intact data obtained through Genepix processing were arranged by low intensity cut-off and normalization, and then were statistically averaged by T-test and verified with p-value. A fold ratio indicates a relative change of transcriptional level by spot intensities of the Hsp22+ flies over control flies for individual gene. Spots with ratio over 1 ($\log_2 > 0$) were considered as up-regulated and spots with ratio below 0.5 ($\log_2 < -1$) were considered as down-regulated. Based on these criteria, 1148 spots (850 genes) were up-regulated in 1-day-old Hsp22+ flies, 1439 spots (937 genes) in 45-day-old flies. 1027, 944 and 1009 spots (686, 610 and 689 genes) were up-regulated at 95%, 90% and 50% survival respectively. As shown in Table 1, the portions of up-regulated spots ranged from 9.8% to 16.9% depending on the tested ages whereas, down-regulated spots were less than 1% under all conditions. Although some genes were commonly up-regulated at all ages, there were no down-regulated genes constantly observed among all different sets of microarray (see Additional data file B, C, D, E, F and G). Therefore, genes are mainly up-regulated in Hsp22 over-expressing flies, suggesting that they have a higher transcriptional level than control flies. In order to confirm the gene expression pattern identified in microarray analysis, *gal4* and *hsp22* genes were examined by RT-PCR (Fig. 5-4). The genes expression pattern measured by RT-PCR was consistent with the pattern of relative fold obtained in microarrays.

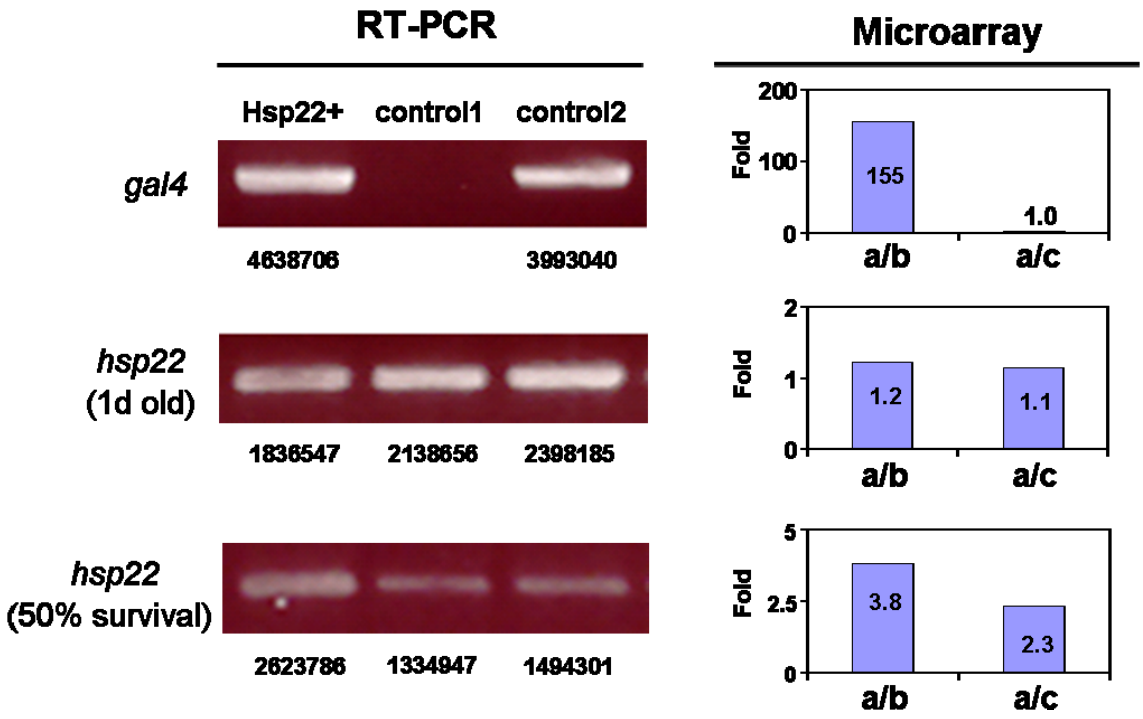

Fig. 5-4. Confirmation of microarray gene data using RT-PCR

With the total RNA used in microarray analysis, expressions of *gal4* and h*sp22* genes were measured by RT-PCR. The RT-PCR (left) and microarray data (right graph) show a relative ratio of *gal4* expression in 1-day-old flies, *hsp22* expression in 1-day-old flies, and *hsp22* expression at 50% survival. The fold indicated here (in microarray data) is a median value among multi spots for each gene of *gal4* and *hsp22*. a, *Hsp22+* (*actin*-Gal4;EP(3)3247) flies; b, control 1 (*cyo*;EP(3)3247) flies c, control 2 (*actin*-Gal4;TM3,*sb*) flies. a/b indicates gene expressing ratio of Hsp22+/control 1 flies, and a/c indicates ratio of Hsp22+/control 2 flies.

5.4.3 More genes are up-regulated in 45-day-old Hsp22 over-expressing flies than in 1 day-old flies

As mentioned above, differences observed at the same chronological age should include both targets of Hsp22 and potential biomarkers of aging that have their time course delayed. Not surprisingly, there are more genes up-regulated in 45 days-old flies over-expressing Hsp22 than in 1 day-old flies (Table 5-1, Fig. 5-5A). Especially, there were at least twice proportion of genes involved in functions such as antioxidant activity (2.4), defense/stress response (3.1), determination of lifespan (2.3), electron transport (5.6), oxidoreductase activity (4.7), phosphorylation (2.8) and proteolysis (2.2) that were up-regulated in 45 days-old Hsp22+ flies comparatively to 1 day-old Hsp22+ flies. Functions related to ribosome (1.1), protein biosynthesis (1.2), transcription regulator activity (1.1) and translation regulation activity (1.3) however, did not show significant changes between 1 and 45 days-old flies (Fig. 5-5A). Therefore, there is higher proportion of gene alteration at 45 days-old flies than at 1 day-old flies.

Table 5-1. Overall changes in gene expression in Hsp22+ flies at different ages

Spots	Number of spots/ % of total spots				
	1 day-old	45 days-old	95% survival	90% survival	50% survival
Total	11480	8496	9811	9668	8375
Up	1148/ 10%	1439/ 16.9%	1027/ 10.5%	944/ 9.8%	1009/ 12%
Down	19/ 0.2%	60/ 0.7%	30/ 0.3%	9/ 0.1%	47/ 0.6%
Not changed	10313/ 89.8%	6997/ 82.4%	8754/ 89.2%	8715/ 90.1%	7319/87.4%

A

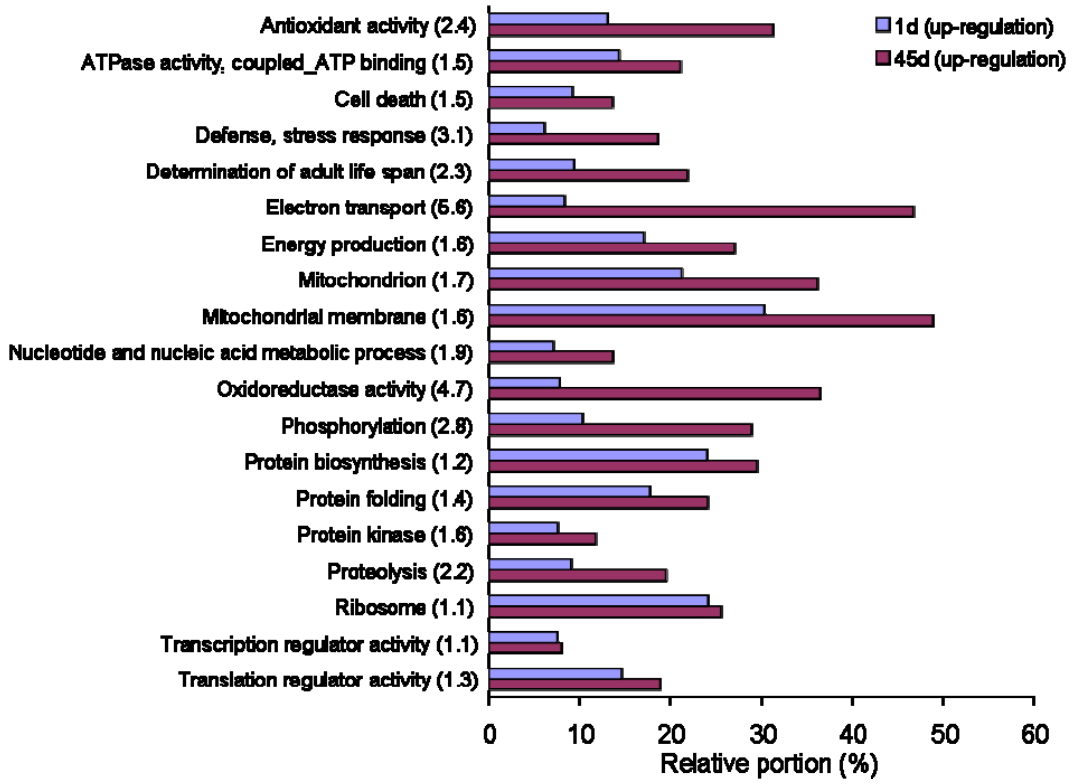

Fig. 5-5. Functions up-regulated in Hsp22+ flies at 1 and 45 days-old

A) Overall up-regulated functions at different chronological ages (day 1 and day 45). The relative proportion means the percentage (%) of up-regulated spots over the total spots number involved in each functional category. This functional analysis was performed according to the classification by Flybase Gene Ontology database. The up-regulated spots of unknown function were not considered. Some gene spots were involved in multiple functions. The number shown in parenthesis indicates a changed rate of the proportion of up-regulated genes during chronological aging from day 1 to day 45.

B

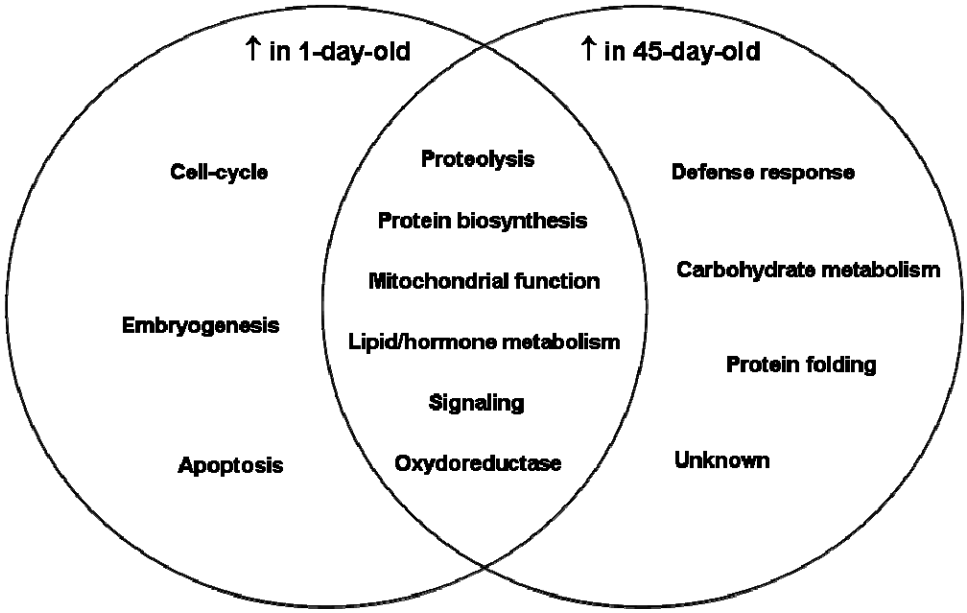

Fig. 5-5. (continued) B) Functions of genes highly up-regulated in Hsp22+ flies at different chronological age (day 1 and day 45). Here, highly up-regulated genes were selected within the top 10% (p-value < 0.01, fold ratio ≥ ≈ 2) of each age and their functions are presented in a Venn diagram.

5.4.4 Genes up-regulated in 1 and 45 days-old Hsp22+ flies share functional similarities

To understand critical differences between Hsp22+ flies and control flies of the same chronological age, we focused our attention on the more highly up-regulated genes and their corresponding functions. Genes ranked within top 10% fold ratio ($p < 0.01$) from total up-regulated genes at each age were considered for further analysis. According to this criteria, 19 genes (22 spots) ranging from 3.8 to 1.9 fold ratio were selected for 1 day-old flies and 43 genes (59 spots) ranging from 5.1 to 2.0 fold ratio were selected for 45 days-old flies. The genes and their functions are presented in Table 5-2. Interestingly, many genes involved in the mitochondrial electron transport chain (*CG3944*, *CG3214*, *CG9172*, *CG5703, CG9172*, *CG7580, CG2249* and C*G6030*), and especially in complex I activity were up-regulated in 45 days-old Hsp22 over-expressing flies. Genes involved in proteolysis such as *CG13095*, *CG12374*, *CG31200*, *CG2145* and *CG4914*, and multiple genes having an oxidoreductase activity were also up-regulated in 45 days-old flies (*CG3360*, *CG10833*, *CG9331*, *CG6910*, *CG3597*, *CG2254* and *CG8453*).

The main functions of the selected genes were put in a Venn-diagram to highlight similarities between 1 day-old and 45 days-old Hsp22+ flies (Fig. 5-5B). Although the genes are not always the same between 1 day-old and 45 days-old data sets, their functional category is related to the same biological processes or molecular functions. Thus, genes that are highly up-regulated in both 1 and 45 days-old Hsp22+ flies are mainly involved in mitochondrial functions, protein biosynthesis, proteolysis, lipid/hormone metabolism, signaling or have an oxidoreductase activity.

Table 5-2. Highly up-regulated genes in 1 and 45 days-old Hsp22+ flies

Gene	Age	Gene ratio	Function	Category
CG6608	1 d	3.8	cation transport, mitochondrial membrane	Mtiochondrial function
CG8743	1 d	3.6	lipid metabolism, calcium channel activity	Lipid/hormone metabolism
baz	1 d	3.3	neuroblast cell division, cytokinesis, oocyte cell fate determination	Embryogenesis
CG7724	1 d	2.8	cholesterol metabolism, oxidoreductase activity	Lipid/hormone metabolism
tiptop	1 d	2.6, 2.7	transcription factor activity	Protein biosynthesis
polo	1 d	2.4, 2.5	protein serine/threonine kinase activity	Signaling
mRpS31	1 d	2.5	mitochondrial small ribosomal subunit	Mitochondrial function
BicD	1 d	2.3, 2.4	chromatin binding, oocyte cell fate determination, mRNA localization	Embryogenesis
mr	1 d	2.4	regulation of cell cycle, mitosis	Cell cycle
CG14715	1 d	2.2	regulation of cell cycle, protein folding, calcium-mediated signaling	Cell cycle
Tim9a	1 d	2.2	mitochondrial inner membrane presequence translocase complex	Mitochondrial function
CG10648	1 d	2.1	protein complex assembly, cell cycle	Cell cycle
Acp32CD	1 d	2.1	hormone activity, negative regulation of female receptivity, post-mating	Lipid/hormone metabolism
hlk	1 d	2.1	induction of apoptosis, lysosome targeting	Apoptosis
EG:103B4.2	1 d	2.1	oxidoreductase activity	Other
RpL27A	1 d	2.0	Ribosomal protein L27A	Protein biosynthesis
qkr58E-1	1 d	1.9	nucleic acid metabolism, apoptosis, spermatogenesis, cell proliferation, RNA binding	Apoptosis
CG32473	1 d	1.9	proteolysis and peptidolysis, glutamyl aminopeptidase activity	Proteolysis
CG8494	1 d	1.9	proteolysis and peptidolysis, ubiquitin-specific protease activity	Proteolysis
CG13095	45 d	4.9-5.1	proteolysis and peptidolysis	Proteolysis
CG12374	45 d	4.9	proteolysis and peptidolysis, carboxypeptidase A activity	Proteolysis
Cyp313a1	45 d	4.3	steroid metabolism, oxidoreductase activity	Lipid/hormone metabolism
CG9468	45 d	3.8	carbohydrate metabolism, hydrolase activity, alpha-mannosidase activity	Carbohydrate melatolism
CG18609	45 d	3.5	lipid metabolism, acyltransferase	Lipid/hormone metabolism
fbp	45 d	3.3	carbohydrate metabolism, fructose-bisphosphatase activity	Carbohydrate melatolism
ATPCL	45 d	3.2	tricarboxylic acid cycle, ATP citrate synthase activity	Mitochondrial function
ND23	45 d	2.9, 3.2	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
Hsp22	45 d	3.0, 3.1	determination of adult life span, protein refolding, response to heat	Defense response
CG10237	45 d	3.0	coenzyme and prosthetic group metabolism, vitamin or cofactor transport	Lipid/hormone metabolism

Table 5-2. (continued)

Gene	Age	Gene ratio	Function	Category
CG31200	45 d	2.9	serine-type endopeptidase activity	Proteolysis
Glt	45 d	2.8, 2.9	calcium ion binding, basement membrane	Signaling
CG3214	45 d	2.7, 2.7	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
Pbprp2	45 d	2.5, 2.7	pheromone binding	Lipid/hormone metabolism
CG9172	45 d	2.1, 2.6	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
CG10184	45 d	2.6	amino acid catabolism, threonine aldolase activity	Proteolysis
CG1583	45 d	2.5	lipid metabolism, phospholipase A2 activity	Lipid/hormone metabolism
CG7580	45 d	2.5	ubiquinol-cytochrome-c reductase activity, mitochondrial electron transport	Mitochondrial function
CG4346	45 d	2.2, 2.5	carbohydrate metabolism, xylulokinase activity	Carbohydrate melatolism
CG2145	45 d	2.4	serine-type peptidase activity	Proteolysis
GstE8	45 d	2.4	Glutathione S transferase E8	Antioxidant
CG32653	45 d	2.4	regulation of transcription, protein binding; zinc ion binding	Protein biosynthesis
CG2249	45 d	2.3	cytochrome-c oxidase activity, respiratory chain complex IV	Mitochondrial function
CG5703	45 d	2.2, 2.3	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
Cyp28d1	45 d	2.3	steroid metabolism, oxidoreductase activity	Lipid/hormone metabolism
CG9331	45 d	2.2, 2.3	carbohydrate metabolism, amino acid biosynthesis, oxidoreductase activity	Carbohydrate melatolism
CG4914	45 d	2.3	proteolysis and peptidolysis, serine-type endopeptidase activity, trypsin activity	Proteolysis
CG6910	45 d	2.2, 2.2	carbohydrate metabolism, nucleic acid metabolism, oxidoreductase activity	Carbohydrate melatolism
CG6673	45 d	2.2	defense response, selenium binding	Defense response
CG4347	45 d	2.0, 2.0, 2.2	polysaccharide metabolism, UTP-glucose-1-phosphate uridylyltransferase activity	Carbohydrate melatolism
BG:DS07851.3	45 d	2.2	regulation of translation, negative regulation of protein biosynthesis	Protein biosynthesis
CG32576	45 d	2.0, 2.1	biological process unknown	Unknown
CG11183	45 d	2.0, 2.1	transmembrane receptor protein serine/threonine kinase signaling pathway	Signaling
fs(1)Yb	45 d	2.1	mitochondrial large ribosomal subunit, constituent of ribosome, protein biosynthesis	Mitochondrial function
CG3597	45 d	2.1	oxidoreductase activity	Oxidoreductase
ATPsyn-d	45 d	2.0, 2.1	proton-transporting ATP synthase complex, coupling factor F(o)	Mitochondrial function
Ppt1	45 d	2.1	protein lipidation, palmitoyl-(protein) hydrolase activity, determination of adult life span	Lipid/hormone metabolism
CG1647	45 d	2.1	monosaccharide metabolism, aldose 1-epimerase activity	Carbohydrate melatolism
CG2254	45 d	2.0	visual perception, oxidoreductase activity	Oxidoreductase
Cyp6g1	45 d	2.0	oxidoreductase activity, response to insecticide, response to DDT	Defense response
CG15093	45 d	2.0	pentose-phosphate shunt, 3-hydroxyisobutyrate dehydrogenase activity	Mitochondrial function
Fkbp13	45 d	2.0	protein folding, peptidyl-prolyl cis-trans isomerase activity, FK506 binding	Protein folding
Mcm3	45 d	2.0	argininosuccinate lyase activity	Unknown

5.4.5 Hsp22+ flies display an increase in genes having an antioxidant activity and involved in proteolysis

Physiological ages represent the same steps in the longevity curve. Thus, flies at the same physiological age have different chronological ages (refer to Figure 3). For example, at 90% survival, which is considered as the entry in the mortality phase, long-lived Hsp22+ flies are approximately 42~ 45 days-old while control flies are 23~ 28 days-old. Figure 5-6A shows the functions related to the genes that are up-regulated in Hsp22+ flies at 95, 90 and 50% survival. Genes having an antioxidant activity and involved in protein folding show the largest differences between each physiological ages tested, with at least three times increase in 50% survival Hsp22+ flies compared to 95% survival Hsp22+ flies. Genes involved in mitochondrial function and determination of adult lifespan, are also more up-regulated with increasing physiological age. In only one case, a functional group of oxidoreductase activity is more represented at 95% survival comparatively to 90 and 50% survival. Indeed, portion of its up-regulated genes at 95% survival are approximately three times larger than those at the other physiological ages. The remaining functions are up-regulated to almost the same extent under all three conditions.

5.4.6 Structural constituents of the ribosome are consistently up-regulated in Hsp22+ flies at all physiological ages tested

As for the chronological age data sets, we next focused our attention on the more highly up-regulated genes and their corresponding functions at each physiological age to uncover critical differences between Hsp22 over-expressing flies and control flies. Therefore, genes ranked within top 10% fold ratio ($p < 0.01$) from total up-regulated genes at each age were considered for further analysis. According to this criterion, 30 genes (43 spots) were selected for 95% survival flies, 27 genes (39 spots) for 90% survival flies and 25 genes (34 spots) for 50% survival flies (Table 5-3). Structural constituents of ribosome are the most up-regulated genes' function in Hsp22+ flies at all physiological ages. Interestingly, five of these genes are consistently observed in the top 10% of genes up-regulated in all three conditions (*CG10423, CG4651, CG7622, CG4111* and *CG8415*). Other genes involved in protein biosynthesis are also up-regulated. No other function seems to be preferentially up-regulated in Hsp22+ flies. One miscellaneous gene is *CG9305*, which is highly up-regulated in Hsp22+ of 95% survival

(gene ratio of 17.4). The molecular function of this gene is known as transcription factor activity and it carries a BDP1 domain. In *Saccharomyces cerevisiae*, BDP1 is one of the three subunits of TFIIIB while in humans it co-purifies with TFIIIC (reviewed in Kassavetis and Geiduschek, 2006) both of which are transcription factors of RNA polymerase III (polIII). The polIII transcription apparatus is devoted to the production of small structural and catalytic RNAs. In *Drosophila*, TRF1/BRF complex is involved in all known classes of PolIII transcription (Isogai et al., 2007), but nothing is uncovered about a possible BDP1 paralogue.

As in the chronological age study, the main functions of the top 10% up-regulated genes were put in a Venn-diagram to highlight similarities between 95, 90 and 50% survival (Fig. 5-6B). Although there is no gene function which is more represented at a given physiological age other than protein biosynthesis, the Venn-diagram has identified genes involved in mitochondrial function, protein biosynthesis, proteolysis, defense response, carbohydrate metabolism, lipid/hormone metabolism and cytoskeleton as being commonly up-regulated at all physiological ages tested. These are reminiscent of some functions obtained in the analysis under the chronological age conditions.

A

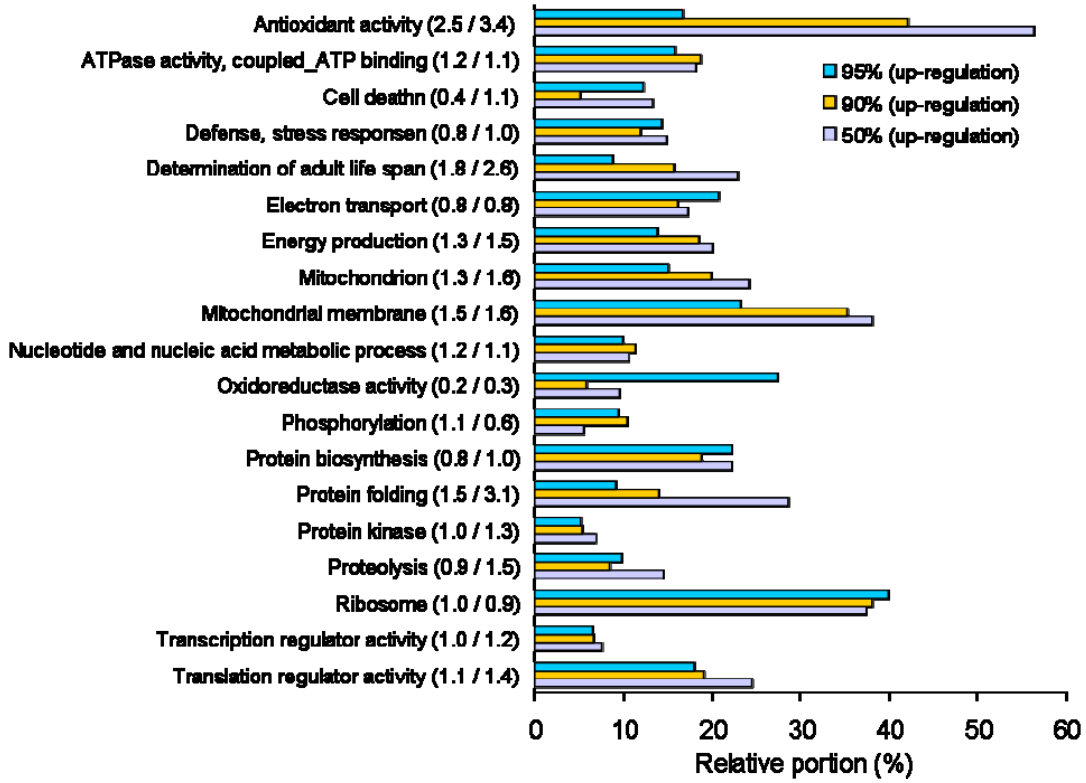

Fig. 5-6. Functions up-regulated in Hsp22+ flies at 95, 90 and 50% survival

A) Overall up-regulated functions at different physiological ages (95%, 90% and 50% survival). The relative proportion means the percentage (%) of up-regulated spots over the total spots number involved in each functional category. This functional analysis was performed according to the classification by Flybase Gene Ontology database. The up-regulated spots of unknown function were not considered. Some gene spots were involved in multiple functions. The numbers shown in parenthesis indicate a changed rate during physiological aging (proportion at 90% survival divided by one at 95% survival / proportion at 50% survival divided by one at 95% survival).

B

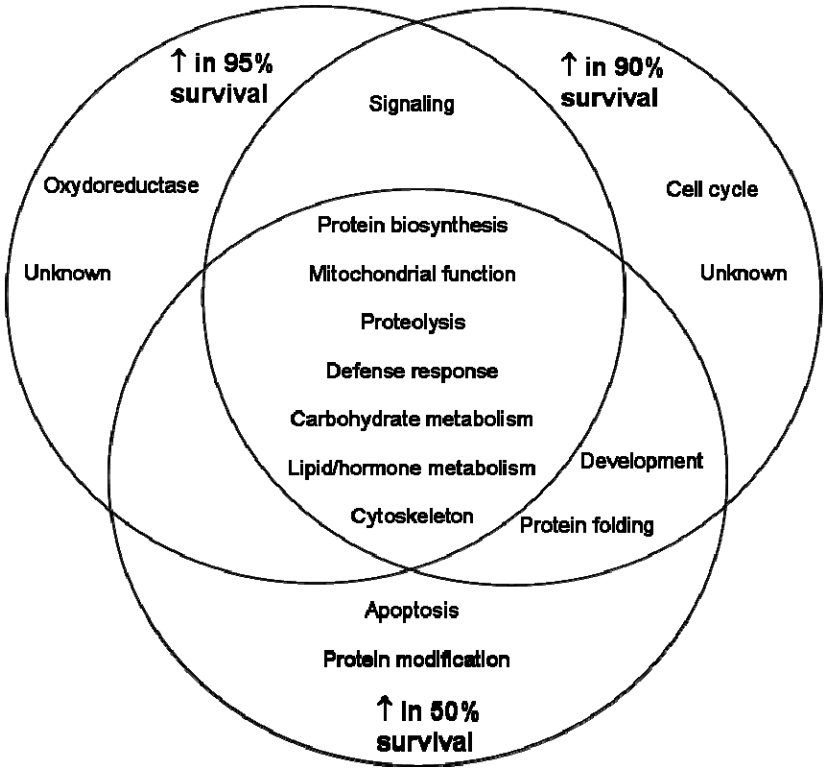

Fig. 5-6. (continued) B) Functions of genes highly up-regulated in Hsp22+ flies at different physiological ages (95%, 90% and 50% survival). Here, highly up-regulated genes were selected within the top 10% (p-value < 0.01, fold ratio ≥ ≈ 2) of each age and their functions are presented in a Venn diagram.

Table 5-3. Highly up-regulated genes in 95%, 90% and 50% survival Hsp22+ flies

Gene	Age	Gene ratio	Function	Category
CG9305	95%	17.4	transcription factor activity	Protein biosynthesis
CG18609	95%	3.1, 3.4	lipid metabolism, acyltransferase activity	Lipid/hormone metabolism
Cyp9b2	95%	3.0	oxidoreductase activity, electron transporter activity	Oxidoreductase
CG6277	95%	2.9	lipid metabolism, triacylglycerol lipase activity	Lipid/hormone metabolism
BcDNA:GH03694	95%	2.6	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
RpL13	95%	2.4	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
RpL36	95%	2.1, 2.4	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG4716	95%	2.3	methylenetetrahydrofolate dehydrogenase activity	Unknown
CG1475	95%	2.3	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Cyp6a23	95%	2.0, 2.3	steroid metabolism, oxidoreductase activity, electron transporter activity	Lipid/hormone metabolism
CG1475	95%	2.2	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
CG13095	95%	2.2, 2.2	proteolysis and peptidolysis	Proteolysis
CG12740	95%	2.2	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
CG17382	95%	2.0, 2.2	intracellular protein transport, G-protein coupled receptor protein	Signaling
CG6910	95%	2.1, 2.2	carbohydrate metabolism, oxidoreductase activity	Carbohydrate metabolism
Act88F	95%	2.1	structural constituent of cytoskeleton, actin filament	Cytoskeleton
sop	95%	2.1, 2.1	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG4111	95%	2.0, 2.1	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG8415	95%	2.1	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG4594	95%	2.1	fatty acid beta-oxidation, dodecenoyl-CoA delta-isomerase activity	Mitochondrial function
fs(1)Yb	95%	2.0, 2.1	structural constituent of ribosome, mitochondrial large ribosomal subunit	Protein biosynthesis
CG9603	95%	2.0	cytochrome-c oxidase activity, mitochondrial electron transport	Mitochondrial function
CG6357	95%	2.0, 2.0	cathepsin L activity	Proteolysis
eIF-4a	95%	2.0, 2.0	RNA helicase activity, translation initiation factor activity, RNA cap binding	Protein biosynthesis
CG10433	95%	2.0	antimicrobial protein homology of seminal fluid	Defense response
CG5708	95%	2.0	transcription from Pol II promoter, transcription regulator activity	Protein biosynthesis
CG3734	95%	2.0	proteolysis and peptidolysis, serine-type peptidase activity	Proteolysis
CG11071	95%	2.0	transcription factor activity	Protein biosynthesis
CG13252	95%	2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis

Table 5-3. (continued)

Gene	Age	Gene ratio	Function	Category
CG8415	95%	2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Cyp4g1	95%	2.0	oxidoreductase activity, electron transporter activity	Oxidoreductase
CG13091	95%	2.0	oxidoreductase activity	Oxidoreductase
BcDNA:GH03694	90%	2.4, 2.4	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
hsp22	90%	2.2, 2.4	determination of adult life span, protein refolding, response to heat	Defense response
RpL36	90%	2.3, 2.4	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG31287	90%	2.2, 2.3	protein folding, protein binding, prefoldin complex	Protein folding
mus304	90%	2.3	DNA damage checkpoint, DNA repair, ATP-dependent DNA helicase activity	Cell cycle
RpS8	90%	2.2	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
desat1	90%	2.2, 2.2	Fatty acid biosynthesis, stearoyl-CoA 9-desaturase activity	Lipid/hormone metabolism
CG4111	90%	2.1, 2.2	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG1475	90%	2.0, 2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Cyt-c-p	90%	2.0	transferring electrons from CoQH2-cytochrome c reductase complex	Mitochondrial function
CG8415	90%	2.0, 2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG6910	90%	2.0	carbohydrate metabolism, nucleic acid metabolism	Carbohydrate metabolism
Act88F	90%	2.0	structural constituent of cytoskeleton, actin filament	Cytoskeleton
RpL13	90%	1.9, 2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Fas2	90%	1.9	tracheal system and skeletal muscle development, neuron differentiation	Development
CG32744	90%	1.9	proteolysis and peptidolysis	Proteolysis
CG7901	90%	1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Ef1α48D	90%	1.9, 1.9	translation elongation factor activity, GTPase activity	Protein biosynthesis
CG6113	90%	1.9	lipid metabolism, triacylglycerol lipase activity	Lipid/hormone metabolism
CG4759	90%	1.9, 1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG2656	90%	1.9	purine nucleotide binding	Unknown
dynactin-su p25	90%	1.9	microtubule associated complex, dynactin complex	Cytoskeleton
Aats-ser	90%	1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
RpS3	90%	1.9	structural constituent of ribosome, DNA-lyase activity, DNA repair	Protein biosynthesis
CG1973	90%	1.9	protein kinase activity	Signaling
CG3532	90%	1.9	structural constituent of cytoskeleton, cell cycle, microtubule binding	Cytoskeleton
sop	90%	1.9, 1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis

Table 5-3. (continued)

Gene	Age	Gene ratio	Function	Category
CG9468	50%	3.0, 3.7	carbohydrate metabolism, hydrolase activity, alpha-mannosidase activity	Carbohydrate metabolism
CG6277	50%	2.2, 3.3	lipid metabolism, triacylglycerol lipase activity	Lipid/hormone metabolism
Hsp22	50%	2.5, 2.5	determination of adult life span, protein refolding, response to heat	Defense response
BcDNA:GH03694	50%	2.3, 2.5	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
GstD9	50%	2.3	defense response, glutathione transferase activity	Defense response
CG8871	50%	2.3	serine-type peptidase activity, proteolysis and peptidolysis, elastase activity	Proteolysis
RpL36	50%	2.1, 2.2	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Mst77F	50%	2.0, 2.1	involved in compaction of Drosophila sperm chromatin	Development
ND23	50%	2.1	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
Hsp27	50%	2.1	protein folding, response to heat	Defense response
RpS3	50%	1.9, 2.1	structural constituent of ribosome, DNA-lyase activity, DNA repair	Protein biosynthesis
CG8415	50%	2.0	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG1647	50%	2.0, 2.0	monosaccharide metabolism, aldose 1-epimerase activity	Carbohydrate metabolism
starvin	50%	2.0	apoptosis, proteolysis	Apoptosis
CG18418	50%	2.0	cation transport, mitochondrial alpha-ketoglutarate/malate transport	Mitochondrial function
sgl	50%	2.0	lipid metabolism, UDP-glucose 6-dehydrogenase activity	Lipid/hormone metabolism
dynactin-su p25	50%	1.9	microtubule associated complex, dynactin complex	Cytoskeleton
CG7170	50%	1.9	chymotrypsin activity, proteolysis and peptidolysis	Proteolysis
CG31528	50%	1.9	protein modification	Protein modification
RpL13	50%	1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
CG5017	50%	1.9, 1.9	protein complex assembly, nucleosome assembly	Protein folding
RpS8	50%	1.9	structural constituent of ribosome, protein biosynthesis	Protein biosynthesis
Act88F	50%	1.9	structural constituent of cytoskeleton, actin filament	Cytoskeleton
CG32744	50%	1.9	proteolysis and peptidolysis	Proteolysis
CG4111	50%	1.9	protein biosynthesis, structural constituent of ribosome	Protein biosynthesis

5.4.7 Protein biosynthesis and mitochondrial functions are commonly increased in Hsp22+ flies of 45 days-old and at 50% survival

Another way to look at primary targets of Hsp22 is to determine commonly up-regulated genes between 45 days-old flies (which correspond to ≈ 50% survival for control 1 and 2 and to ≈ 90% survival for Hsp22+ flies) and 50% survival flies (which correspond to ≈ 45 days for control 1 and 2 and to ≈ 65 days for Hsp22+ flies). This comparison is based on the assumption that Hsp22 simply extends lifespan and changes the normal time course of gene expression. Therefore, long-lived flies of the same chronological age should include both targets of Hsp22 as well as potential biomarkers of aging that scale with "physiological age" (delayed by ≈ 30% in their time course) while long-lived flies of the same physiological age should include both targets of Hsp22 as well as alterations that do not simply represent a delay in normal aging patterns (Fig. 5-3). Genes altered consistently in both the same chronological age (45 days-old) and the same physiological age (50% survival) should represent the primary targets of Hsp22. According to this criterion, 87 genes (121 spots) were commonly up-regulated (Table 5-4). 17 genes involved in protein biosynthesis (of which 9 are structural constituent of the ribosome and 8 are transcription and translation regulators) were commonly up-regulated in 45 days-old and 50% survival Hsp22+ flies as well as 21 genes involved in mitochondrial function. Interestingly, among those genes, 6 are part of the complex I, and 4 others are part of the ATP pump (complex V). Genes linked to defense response (9) and proteolysis (9) were also up-regulated.

Table 5-4. Genes up-regulated in both 45 days-old and 50% survival Hsp22+ flies

Name	Gene ratio		Function	Category
	Day45	50% survival		
CG3534	2.2	1.6	carbohydrate metabolism	Carbohydrate metabolism
CG9468	3.8, 4.0	3.0, 3.7	carbohydrate metabolism, hydrolase activity, hydrolyzing N-glycosyl compounds	Carbohydrate metabolism
CG6910	2.2	1.5, 1.7	carbohydrate metabolism, nucleic acid metabolism, oxidoreductase activity	Carbohydrate metabolism
CG32444	2.1, 2.4	2.0	monosaccharide metabolism	Carbohydrate metabolism
Eb1	1.5	1.2	regulation of cell cycle, microtubule associated complex, mitotic spindle positioning and orientation	Cell cycle
CG14995	1.6	1.2	salivary gland cell death, autophagic cell death	Cell death
CG3532	1.5, 1.9	1.6, 1.7	structural constituent of cytoskeleton, cell cycle, mitosis, microtubule binding	Cytoskeleton
CG1681	1.6	1.1	defense response, response to toxin, glutathione transferase activity	Defense response
GstS1	1.9, 2.3	1.6, 1.7	glutathione transferase activity, glutathione peroxidase activity, defense response	Defense response
Jafrac1	1.7	1.2, 1.3	glutathione/ thioredoxin peroxidase activities, cell redox homeostasis, oxygen/ROS metabolism	Defense response
Hsp22	3.0, 3.1	2.5	hsp22 determination of adult life span , protein refolding, response to heat	Defense response
Jafrac2	1.7	1.2	oxygen and reactive oxygen species metabolism, defense response, thioredoxin peroxidase activity	Defense response
Fkbp13	1.6	1.2	protein folding, peptidyl-prolyl cis-trans isomerase activity, FK506 binding	Defense response
CG7768	1.6	1.3	protein folding, protein targeting, defense response, peptidyl-prolyl cis-trans isomerase activity	Defense response
CG7945	1.3	1.3	protein folding, unfolded protein binding	Defense response
Cyp6g1	1.9, 2.0	1.3, 1.4	response to insecticide, response to DDT, response to organophosphorus	Defense response
CG13898	1.9	1.5	calmodulin binding, ectoderm development, calcium-mediated signaling, calcium ion binding	Development
Ald	1.7	1.5	fructose-bisphosphate aldolase activity, glycolysis, mesoderm development	Development
JanA	2.0	1.6	sex differentiation	Development
aret	1.6, 1.9	1.5, 1.6	spermatid development, oogenesis, negative regulation of oskar mRNA translation	Development
fax	1.3	1.5	transmission of nerve impulse, axonogenesis	Development
Hdc	1.5	1.4	transmission of nerve impulse, histidine decarboxylase activity, eye photoreceptor development	Development
Mst35Bb	2.0	1.5, 1.8	DNA binding	DNA binding
CG8368	1.7	1.5	nuclease activity, exoribonuclease activity, nucleic acid metabolism	DNA metabolism
beat-Vb	1.7, 1.9	1.1, 1.2	extracellular	Extracellular
Gs2	1.7	1.2	glutamate catabolism, neurotransmitter receptor metabolism, cytoplasm, glutamine biosynthesis	Glutamate catabolism
CG18371	1.6	1.5	phosphoric monoester hydrolase activity	Hydrolase
Fer1HCH	1.5, 1.6	1.3	iron ion homeostasis, ferritin complex	Iron homeostasis
CG6277	2.1, 2.2	2.2, 3.3	lipid metabolism, triacylglycerol lipase activity	Lipid metabolism
PbprpR	2.5	1.2	pheromone binding, extracellular, phenylalkylamine binding	Lipid/hormone metabolism
desat1	2.1	1.4	stearoyl-CoA 9-desaturase activity, fatty acid biosynthesis	Lipid/hormone metabolism
CG6404	1.4	1.3	carrier activity, cytochrome c oxidase biogenesis, protein transporter activity	Mitochondrial function
SesB	1.5	1.3, 1.4	cation transport, ADP antiporter activity, mitochondrial inner membrane	Mitochondrial function
CG2249	2.3, 2.7	1.6	cytochrome-c oxidase activity, complex IV, cytochrome c to oxygen	Mitochondrial function
wal	1.6, 1.7	1.3	electron transport, oxidative phosphorylation, electron transfer flavoprotein complex	Mitochondrial function

Table 5-4. (continued)

Name	Gene ratio		Function	Category
	Day45	50% survival		
CG4692	2.1	1.3	electron transport, proton-transporting ATP synthase complex, coupling factor F(o)	Mitochondrial function
Tpi	1.3	1.4	gluconeogenesis, glycolysis, pentose-phosphate shunt, triose-phosphate isomerase activity	Mitochondrial function
Tom7	1.3	1.3	mitochondrial outer membrane translocase complex, protein-mitochondrial targeting	Mitochondrial function
CG12079	1.8	1.3	NADH dehydrogenase activity, complex I	Mitochondrial function
ND23	2.9-3.8	1.6, 2.1	NADH dehydrogenase activity, complex I	Mitochondrial function
CG5703	2.2, 2.3	1.3	NADH dehydrogenase activity, complex I	Mitochondrial function
CG9172	2.1, 2.6	1.6	NADH dehydrogenase activity, complex I	Mitochondrial function
Pdsw	1.4	1.4	NADH dehydrogenase activity, respiratory chain complex I	Mitochondrial function
CG3214	2.7	1.5, 1.6	NADH dehydrogenase activity, respiratory chain complex I, NADH to ubiquinone	Mitochondrial function
CG6543	1.7	1.4	oxidoreductase activity, enoyl-CoA hydratase activity, fatty acid beta-oxidation	Mitochondrial function
CG8036	1.6	1.3	pentose-phosphate shunt, transketolase activity	Mitochondrial function
CG3321	1.7, 1.8	1.4	proton-transporting ATP synthase complex, coupling factor F(o) , hydrogen-exporting ATPase activity	Mitochondrial function
ATPsyn-d	2.0, 2.1	1.5, 1.6	proton-transporting ATP synthase complex, coupling factor F(o) hydrogen-exporting ATPase activity	Mitochondrial function
ATPsyn-b	1.5, 1.6	1.3	proton-transporting ATP synthase complex, coupling factor F(o) hydrogen-exporting ATPase activity	Mitochondrial function
SdhB	1.8	1.2	respiratory chain complex II, TCA cycle, succinate dehydrogenase (ubiquinone) activity	Mitochondrial function
Cyt-c-d	1.6	1.3	transferring electrons from CoQH2-cytochrome c reductase complex, cytochrome c oxidase activity	Mitochondrial function
Cyt-c-p	1.9	1.4	transferring electrons from CoQH2-cytochrome c reductase complex, cytochrome c oxidase activity	Mitochondrial function
pigeon	1.5	1.2	associative, olfactory learning	Olfactory learning
CG3609	1.8	1.7	oxidoreductase activity	Oxydoreductase
CG3699	1.8	1.4	oxidoreductase activity, acting on CH-OH group of donors	Oxydoreductase
mRpL37	2.0, 2.1	1.5, 1.6	structural constituent of ribosome, mitochondrial large ribosomal subunit	Protein biosynthesis
RpL35	1.6, 1.7	1.7, 1.9	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
RpL27	1.6	1.5	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
RpL22	1.4	1.5	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
RpL11	1.5	1.8	structural constituent of ribosome, cytosolic large ribosomal subunit	Protein biosynthesis
RpS15Ab	1.4	1.4	structural constituent of ribosome, cytosolic small ribosomal subunit	Protein biosynthesis
RpS3A	1.3	1.5	structural constituent of ribosome, cytosolic small ribosomal subunit	Protein biosynthesis
RpS19a	1.3	1.5	structural constituent of ribosome, cytosolic small ribosomal subunit	Protein biosynthesis
Sop	1.2, 1.3	1.6	structural constituent of ribosome, cytosolic small ribosomal subunit	Protein biosynthesis
CG5793	1.7	1.3	amino acid metabolism, amino acid catabolism, |lyase activity, isomerase activity	Proteolysis
Prosβ3	1.4	1.4, 1.5	ATP-dependent proteolysis, ubiquitin-dependent protein catabolism, 20S proteasome beta3 su	Proteolysis
Jon66Cii	1.6, 1.8	1.6, 1.9	chymotrypsin activity, proteolysis and peptidolysis	Proteolysis
CG1137	1.6	1.7	peptidase activity	Proteolysis
CG17302	3.7	2.2	peptidase activity, proteasome core complex , ubiquitin-dependent protein catabolism	Proteolysis
CG8564	1.7	1.5	proteolysis and peptidolysis, metallocarboxypeptidase activity	Proteolysis

Table 5-4. (continued)

Name	Gene ratio		Function	Category
	Day45	50% survival		
Spn43Ab	1.6, 1.7	1.6, 1.7	proteolysis and peptidolysis, serine-type endopeptidase inhibitor activity	Proteolysis
Jon25Biii	2.0	1.7	serine-type peptidase activity, proteolysis and peptidolysis, elastase activity	Proteolysis
Ubi-p63E	1.2	1.2	ubiquitin-dependent protein catabolism, regulation of transcription, ATP-dependent proteolysis	Proteolysis
CG6409	1.7, 1.8	1.4, 1.5	endoplasmic reticulum, GPI anchor biosynthesis	Reticulum endoplasmic function
Pof	1.5	1.4	autosome, RNA binding	RNA binding
CG4546	1.5	1.5	amino acid kinase activity, arginine kinase activity	Signaling
Vimar	1.5	1.2	guanyl-nucleotide exchange factor activity, Ral interactor activity	Signaling
Tsp3A	2.5	1.7	receptor binding, signal transduction, cell-cell adhesion, integral to membrane	Signaling
alc	1.4	1.4	receptor signaling protein serine/threonine kinase activity, AMP-activated protein kinase activity	Signaling
tai	1.4	1.5	border cell migration, transcription coactivator activity, regulation of transcription	Transcription/translation regulation
bic	1.3, 1.4	1.7	regulation of transcription from Pol II promoter, nascent polypeptide-associated complex	Transcription/translation regulation
Dcp1	2.0, 2.1	1.3	regulation of transcription from Pol II promoter, signal transduction, serine/threonine kinase receptor	Transcription/translation regulation
CG5708	1.3	1.4	regulation of transcription from Pol II promoter, transcription regulator activity	Transcription/translation regulation
Nap1	1.5	1.3	regulation of transcription, DNA-dependent, histone binding	Transcription/translation regulation
mtTFB1	2.1	1.3	rRNA metabolism, rRNA (adenine) methyltransferase activity	Transcription/translation regulation
CG31159	1.2, 1.4	1.3	translation elongation factor activity, mitochondrion	Transcription/translation regulation
CG10306	1.1	1.4	translation initiation factor activity, eukaryotic translation initiation factor 3 complex	Transcription/translation regulation

5.4.8 Genes displaying the same variation in transcripts level during aging as *hsp22* are involved in mitochondrial function or have a protein kinase activity

We next used a two way-ANOVA similarity analysis through the genome-wide profiling to identify genes showing a similar pattern of transcript level through lifespan. Five genes showing similar transcriptional profile to the one of *hsp22* during chronological and physiological aging were identified (Fig. 5-7A, B). These genes are involved in mitochondrial respiratory complex enzyme activity such as NADH dehydrogenase (*CG3214* and *ND23*) and cytochrome-c oxidase (*CG2249*). *CG32616* (stellate 12D orphon) is involved in spermatogenesis and has a casein kinase II (CK2) domain. A gene of unknown function, *CG14757* is involved in the clustering as showing similar transcriptional profile to *hsp22*.

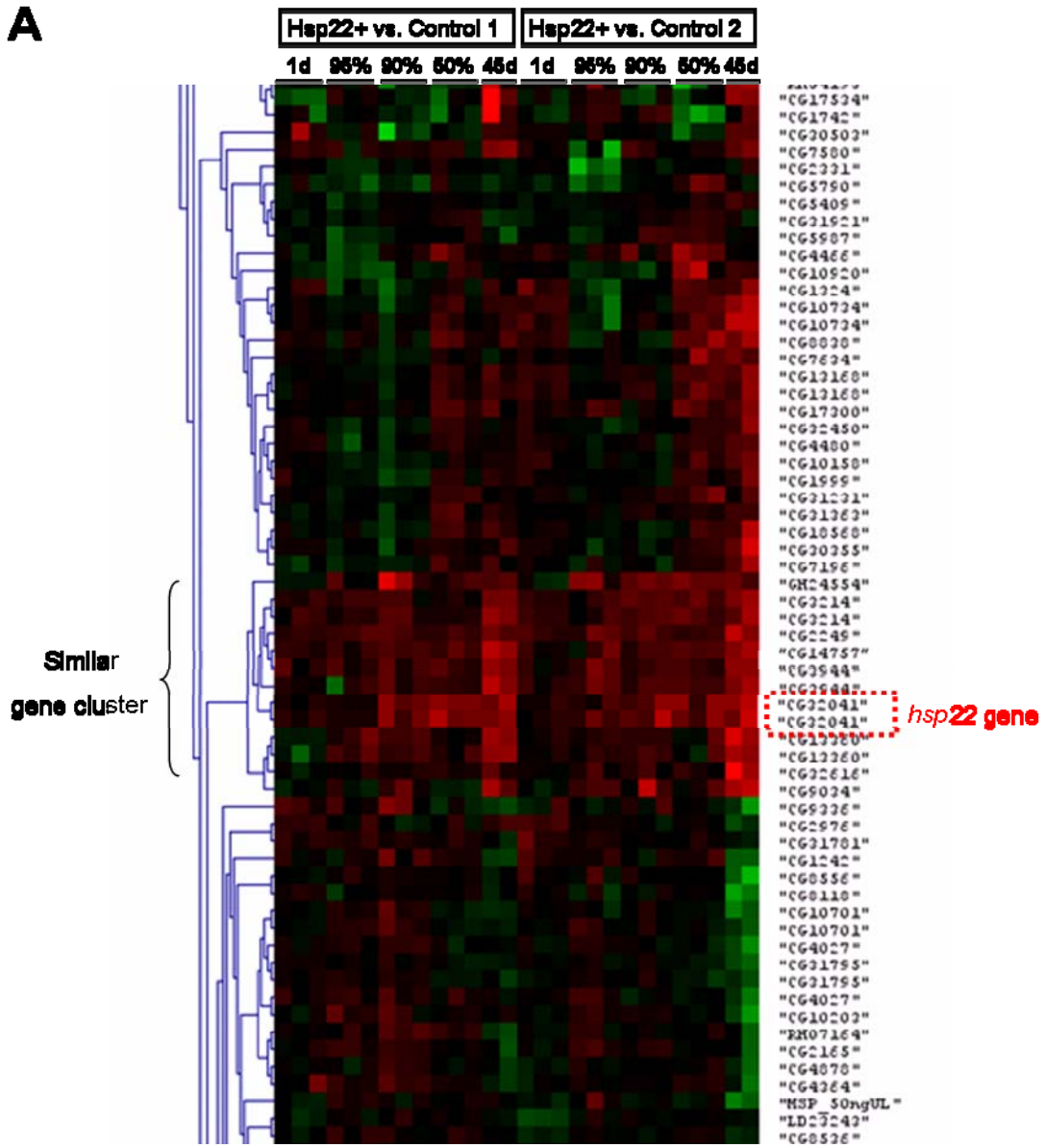

Fig. 5-7. Five genes display a similar pattern of transcriptional profile to the one of *hsp22*

A) Hierarchical gene clustering image,

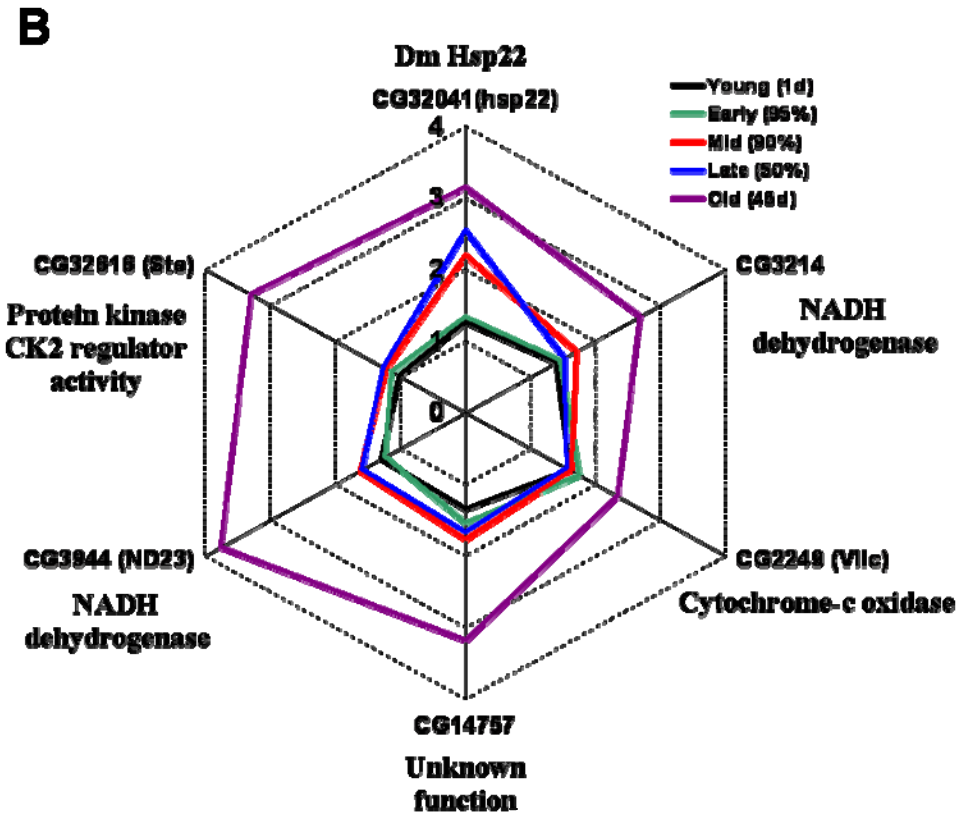

Fig. 5-7. (continued) B) Geometric graph showing a similar pattern of up-regulation with *hsp22*. Genes were screened by using two way-ANOVA similarity analysis through the genome-wide gene profile. Mean value of fold ratio was obtained from 4-6 experiments and 2 different controls and applied to the geometric graph.

5.4.9 Heat shock response genes

The pattern of relative transcriptional level of heat shock response genes was investigated as shown in Fig. 5-8, to see possible correlation between *hsp22* and other *hsp* transcripts. As expected, *hsp22* gene was up-regulated with an increased ratio in both chronological and physiological aging procedures (Fig. 5-8A). In our experiment, the transcriptional expression of *hsp22* gene in the Hsp22+ flies is due to two factors: the endogenous response to age-dependent stresses and the exogenous activation from the GAL4/UAS system. Control flies however, have only the endogenous age-dependent transcriptional activation of Hsp22. In comparison to the up-regulation of *hsp22*, other hsp genes like *hsp23* and *hsp83* did not show significant differences in transcriptional level (Fig. 5-8B, F). The expressions of *hsp26*, *hsp27* and *hsp40*, however, were approximately 2-fold up-regulated at 50% survival and slightly increased at 45 days (Fig. 5-8C, D & E). This can be influenced either by Hsp22 over-expression or by the delayed aging parameters. Hsp22+ flies however, do not result in a universal up-regulation of *hsps*, which can exclude a possible influence of these genes products on the transcriptional profiling analysis.

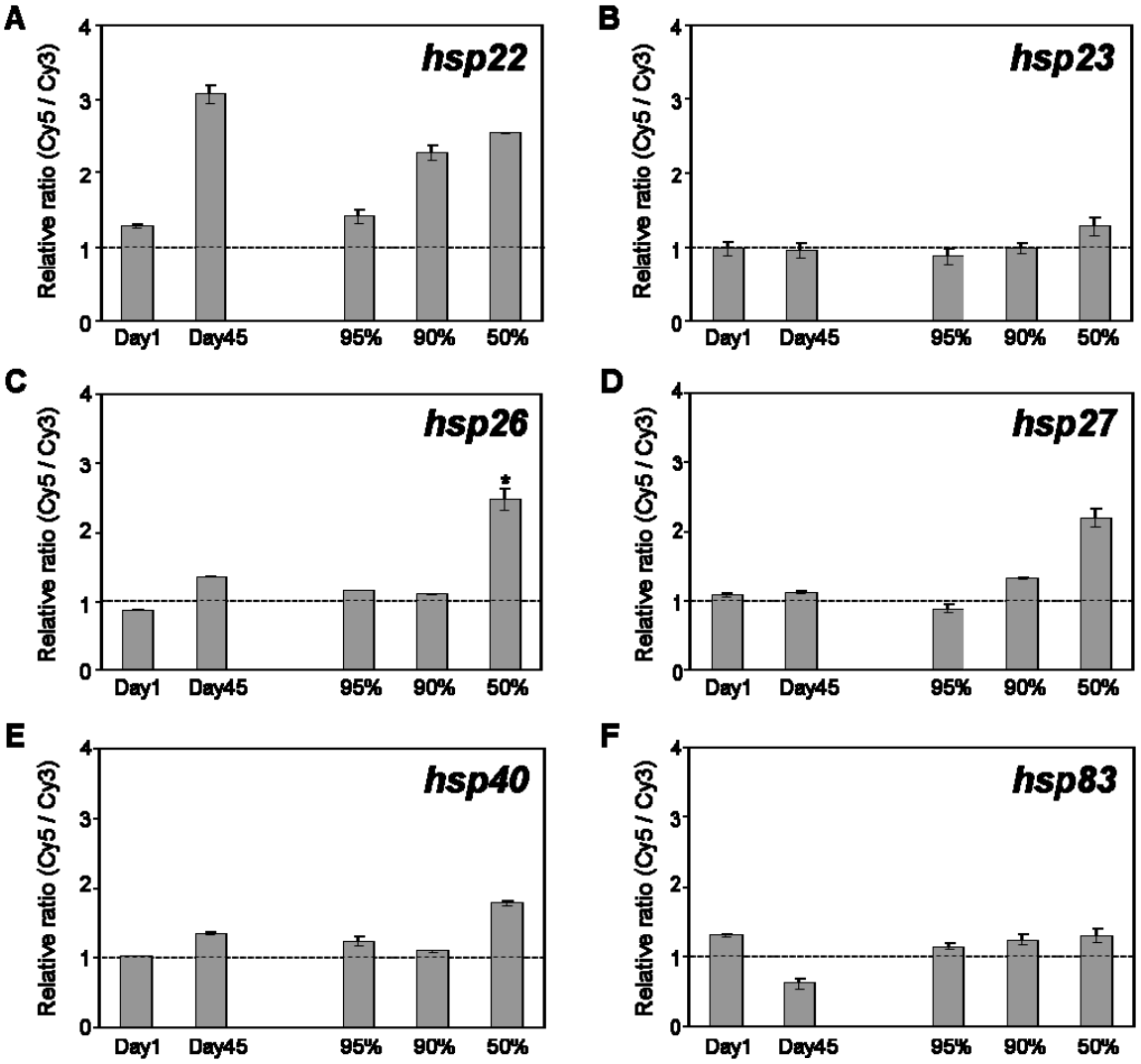

Fig. 5-8. Relative ratio of *hsp* transcripts in Hsp22+ flies versus controls flies

RNA from Hsp22+ flies was labeled with fluorescent dye Cy5-dCTP, and RNA from control was labeled with Cy3-dCTP. Relative ratio reflects Cy5 intensity (Hsp22+ flies) divided by Cy3 intensity (control flies), using normalized spot intensity. Error range indicates standard deviation for mean ratio value from 4-6 experiments and 2 different controls. *: relative ratio was calculated using raw normalized data including low intensity spots. A: transcripts of *hsp22*, B; *hsp23*, C; *hsp26*, D; *hsp27*, E; *hsp40* (dnaJ-like), F; *hsp83*.

5.5 Discussion

5.5.1 The four main functions up-regulated in Hsp22+ flies are usually down-regulated in the aging process

Flies ubiquitously over-expressing the endogenous Hsp22 gene using GAL4/UAS system have a ≈ 30% increase in mean lifespan comparatively to control flies similar to its over-expression in motorneurons (Morrow et al., 2004a). We have taken three different approaches to analyze our microarray data and identify genes that could be related to Hsp22 and its beneficial effect during aging. We have performed comparisons using either flies of the same chronological age, flies of the same physiological age or flies of 45 days-old/50% survival. The three methods yielded different gene sets but there were four main functions associated to these genes in all three comparisons. Hence genes involved in protein biosynthesis, mitochondrial function, defense response and proteolysis were all found to be up-regulated in Hsp22 over-expressing flies.

Multiple studies using transcriptional profiling have been performed in the past decade to unveil the signature of aging. From these studies it stands out that the aging process is shared among organisms and that it involves changes in transcription profile of 15 to 25% of the genes (McCarroll et al., 2004; Pletcher et al., 2002; Zou et al., 2000). An up-regulation of genes involved in the immune system (antibacterial response) and a down-regulation of genes involved in reproduction have been widely observed in aging studies (Lai et al., 2007; Girardot et al., 2006; Zou et al., 2000; Landis et al., 2004). Interestingly, these later genes did not come out in our experiments meaning that there are no or only small differences in the transcription profile of these genes between Hsp22+ and control flies and suggesting that the beneficial effect of Hsp22 is not linked to these functions.

5.5.2 Hsp22+ flies show an up-regulation of genes involved in mitochondrial function

Microarray experiments on aging *Drosophila* have shown an age-related decrease in gene transcripts linked to oxidative phosphorylation and other mitochondrial functions such as

proton transport and TCA cycle energy pathway (Kim et al., 2005; Pletcher et al., 2002; Zou et al., 2000; Girardot et al., 2006; McCarroll et al., 2004; Landis et al., 2004).

Due to their proximity to the electron transport chain, mitochondria are particularly susceptible to damages induced by ROS, the by-product of energy production. Studies carried out in different organisms have unveiled mitochondria morphological alterations associated with aging and a consequent decline in the bioenergetic capacity with age, especially in energy-demanding tissues (Trounce et al., 1989; Yen et al., 1989; Wallace, 1992; Ferguson et al., 2005). In aging flies, the level of transcripts of mitochondrial genes encoded either in the nucleus or in the mitochondria, and involved in the electron transport chain and the TCA cycle, has been shown to be decreased (Schwarze et al., 1998; Calleja et al., 1993; Dubessay et al., 2007; reviewed in Morrow and Tanguay, 2008). Accordingly, the level of mitochondrial small (12S) and large (16S) ribosomal RNA transcripts has been shown to be reduced by 60-80% in aging flies (Calleja et al., 1993; Dubessay et al., 2007), as well as the nuclear genes encoding the mitochondrial transcription factors TFAM, TFB1, TFB2 and DmTTF which are essential for the maintenance and expression of mtDNA (Dubessay et al., 2007), suggesting a reduction of all transcripts encoding mitochondrial functions. The decrease in mitochondrial transcripts is not always correlated with a decrease in protein level and there is still a debate on the correlation between the metabolic rate of mitochondrial energy production and lifespan in *Drosophila* (Hulbert et al., 2004).

Long-lived Hsp22+ flies display an up-regulation of transcripts involved in mitochondrial function. Interestingly, transcripts of complex I and V of the electron transport chain are over represented in our experiment, especially in the 45 days-old/50% survival comparison. Complex I is known to be highly thermosensitive and has been shown to be protected by Hsp27 in plants and in mouse cells (Downs et al., 1999; Downs and Heckathorn, 1998). The availability of transcripts involved in mitochondrial function suggests that mitochondrial homeostasis is better preserved in Hsp22+ flies. We are presently examining if the transcriptional level of mitochondrial genes in Hsp22+ flies is consistent with protein levels and mitochondrial complex activities.

5.5.3 Hsp22+ flies display an increase in transcripts of genes involved in protein biosynthesis

Slowing protein synthesis is one of the most commonly observed biochemical changes during aging. A decline in the efficiency and accuracy of ribosomes, and a decrease in the amount and activity of elongation factors have been observed in flies (Rattan, 1996). As mentioned above, in *Drosophila*, aging is associated with a decrease in the steady-state level of mitochondrial transcripts (encoded either in mitochondria or in the nucleus) and mitochondrial transcriptional factors (Calleja et al., 1993; Dubessay et al., 2007).

Rates of protein synthesis and activities of key mRNA translation factors decline with age in a variety of organisms (Rattan, 1996) and certain mutants for genes involved in ribosomal function also display a defective developmental rate (Lawrence et al., 1986; Duffy et al., 1996; Saeboe-Larssen et al., 1998). In *Drosophila melanogaster* an age-related decrease in polyribosome levels has been observed but it is not clear if such an alteration is a consequence of reduced mitochondrial function and energy production adaptation (Webster and Webster, 1983).

Long-lived Hsp22+ flies clearly show an up-regulation of transcripts of genes involved in protein biosynthesis. Especially, mitochondrial ribosomal subunit genes like *mRpS31*, *fs(1)Yb*, and *mRpS7*, and translational initiation factor and RNA cap binding genes like *eIF-4a*, *eIF-5A*, *eIF-4E*, *eIF5*, and *Ef1α48D*, which show a moderate but constant up-regulation in Hsp22+ flies. Moreover, many structural constituents of ribosomes are up-regulated in Hsp22+ flies in the chronological age and 45 days-old/50% survival comparisons. Interestingly, 12 genes of this category are up-regulated in all age conditions tested (Table 5-5). The over representation of structural constituent of ribosomes could partly explain why Hsp22+ flies display mostly an up-regulation of transcripts.

5.5.4 Hsp22+ flies display an up-regulation of transcripts of genes involved in proteolysis

With age, there is an accumulation of damaged proteins, which could be due to a decreased efficiency of the protein repair and removal systems (Berlett and Stadtman, 1997; Friguet et al., 2000; Petropoulos and Friguet, 2005; Brégégère et al., 2006), but this is now debated. Moreover, there is a lack of consensus on the regulation of genes involved in proteolysis in

genome-wide experiments on *Drosophila* aging, for example in the case of serine protease, some genes being down-regulated while others are not (Zou et al., 2000; Landis et al., 2004).

Hsp22+ flies display a conserved up-regulation of genes involved in proteolysis and ubiquitin cycling, such as *jon66Cii*, *ubiquitin-5E*, *pros25*, *prosα7* and *prosß3* (Table 5-5). Moreover, some endopeptidases such as *Ser99Dc*, *Ser6*, *CG4914* and *CG13095* tend to be up-regulated in Hsp22+ flies.

5.5.5 Hsp22+ flies display an up-regulation of genes involved in defense response

Under conditions of stress caused by the aging process, there is an accumulation of misfolded and aggregated proteins. This is partly due to post-translational modification by ROS. The cell has developed a number of antioxidant defense systems including superoxide dismutase, the peroxidases, and the glutathione redox cycle. However, the activity of these enzymes decreases during aging, which leads to the accumulation of ROS-induced protein damages. Heat shock proteins (Hsps) are part of the cell defense system and are involved among others in refolding of misfolded proteins and targeting of damaged proteins to the proteasome (Schmitt and Langer, 1997; Ranfold et al., 2000). Hsps can also protect cells against the vicious effects of protein aggregates as demonstrated in neurodegenerative diseases studies (Poon et al., 2004). Hsp22 is a sHsp displaying a chaperone-like activity *in vitro* (Morrow et al., 2006). Therefore, its presence in fly mitochondria could contribute to a reduction in the amount of misfolded proteins. Furthermore, as shown in Table 5-5, other genes involved in protein folding activity are up-regulated, suggesting a better system to avoid accumulation of damaged proteins in Hsp22+ flies.

Glutaredoxin/glutathione/glutathione reductase and thioredoxin/thioredoxin reductase systems are other constituents of the cell defense system. They reverse the oxidation of protein disulfide bond and cysteine sulfenic acids (Holmgren et al., 2005). Genes acting on glutathione S transferase S1 (*CG8938*) and thioredoxin/glutathione peroxidase (*CG1633, CG6888* and *CG12013*) are frequently up-regulated in Hsp22+ flies in mostly all data set (Additional data file H). Interestingly, Glutathione S transferase S1 is known to be almost exclusively localized on the thin filaments of the indirect flight muscles (IFMs) (Clayton et al., 1998) in interaction with troponin-H (TnH) (Bullard et al., 1988) where it would protect

muscle or neuronal tissues from deleterious effects of oxidative stress (Singh et al., 2001). Over-expression of Glutathione S transferase S1 using a ubiquitous driver has been shown to increase *Drosophila* lifespan (Seong et al., 2001; Toba and Aigaki, 2000).

Table 5-5. Constantly up-regulated genes and their functions in Hsp22 over-expressing flies at all chronological and physiological ages

Gene name	Chromosome	Function and product	Day1		Day45		95%		90%		50%	
			fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.
Protein folding												
hsp22	3L	Heat shock protein 22	1.3	0.009	3.1	0.003	1.3	0.010	2.2	0.001	2.5	0.000
dod	X	Epidermal growth factor receptor signaling pathway, protein folding	1.1	0.041	1.6	0.057	1.5	0.001	1.4	0.057	1.4	0.017
CG7945	3L	Protein folding	1.1	0.043	1.2	0.083	1.1	0.151	1.3	0.017	1.3	0.004
CG31287	3R	Protein folding, binding	1.1	0.022	1.3	0.020	1.2	0.058	1.2	0.000	1.4	0.000
Lipid metabolizm/hormone activity												
CG15219	2L	Accessory gland protein genes and testis-expressed genes (Wagstaff 2005)	1.4	0.003	1.8	0.077	1.7	0.047	1.5	0.005	1.9	0.002
Acp53Ea	2R	Accessory gland-specific peptide 53Ea, sperm displacement, hormone activity	1.4	0.025	1.6	0.046	2.1	0.018	1.5	0.006	1.2	0.099
Acp53C14a	2R	Accessory gland-specific peptide 53C14a	1.3	0.020	1.5	0.087	2.2	0.033	1.4	0.002	1.2	0.064
Proteasome/proteolysis												
Pros25	3R	Proteasome 25kD subunit, ATP-dependent proteolysis	1.2	0.034	1.3	0.073	1.3	0.004	1.1	0.035	1.2	0.047
Prosα7	2R	Proteasome α7 subunit, proteolysis, ubiquitin-dependent protein catabolism	1.5	0.002	1.3	0.010	1.3	0.008	1.1	0.083	1.2	0.055
Prosβ3	3R	Proteasome β3 subunit, ATP-dependent proteolysis	1.2	0.092	1.4	0.027	1.5	0.000	1.3	0.000	1.5	0.001
Jon66Cii	3L	Proteolysis and peptidolysis, chymotrypsin activity	1.2	0.074	1.6	0.035	2.0	0.020	1.7	0.000	1.6	0.023
Ubiquitin-5E	X	Proteolysis, protein modification	1.4	0.071	1.3	0.060	1.9	0.005	1.6	0.004	1.6	0.001
Oxidative phosphorylation/mitochondria related funtion												
CG2249	2R	Cytochrome-c oxidase (complex IV)	1.6	0.000	2.3	0.005	1.8	0.001	1.6	0.002	1.6	0.000
CG17903	2L	Electron transporter (complex III, IV)	1.5	0.000	1.7	0.088	1.6	0.005	2.0	0.001	1.2	0.198
ATPsyn-d	3R	Hydrogen-transporting ATPase activity (complex V)	1.1	0.386	2.1	0.007	1.4	0.009	1.5	0.011	1.6	0.001

Table 5-5. (continued)

Gene name	Chromosome	Function and product	Day1		Day45		95%		90%		50%	
			fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.
ATPsyn-β	4	Hydrogen-transporting ATPase activity (complex V), ATP synthase β	1.6	0.003	1.8	0.041	1.8	0.001	1.7	0.000	1.4	0.015
CG3321	3R	Hydrogen-transporting ATPase activity (complex V)	1.5	0.002	1.7	0.025	1.6	0.031	1.3	0.017	1.4	0.011
ND23	3R	NADH:ubiquinone reductase 23kD subunit precursor (complex I)	1.3	0.013	2.9	0.000	1.7	0.006	1.7	0.002	1.6	0.000
CG3214	2L	NADH dehydrogenase (complex I)	1.4	0.000	2.7	0.001	1.5	0.009	1.7	0.008	1.5	0.012
CG8844	2L	NADH dehydrogenase (complex I)	1.2	0.037	1.4	0.029	1.4	0.049	1.4	0.015	1.4	0.013
CG18809	X	Cytochrome-c oxidase (complex IV)	1.1	0.022	1.5	0.115	1.2	0.094	1.3	0.000	1.3	0.000
CG16944	X	ATP:ADP antiporter activity (complex V)	1.6	0.001	1.5	0.003	1.8	0.004	1.5	0.006	1.4	0.001
RE48563	3R	ATPsyn-d homolog, hydrogen-exporting ATPase activity (complex V)	1.2	0.027	1.7	0.006	1.2	0.040	1.5	0.002	1.4	0.018
RE74025	3L	Mpcp homolog, mitochondrial phosphate carrier protein	1.3	0.027	1.2	0.110	2.0	0.008	1.6	0.012	1.7	0.006
CG8226	2R	Protein-mitochondrial targeting, protein translocase activity, Tom7	1.2	0.015	1.3	0.031	1.3	0.045	1.2	0.038	1.3	0.001
Protein biosynthesis/translational regulation												
CG6547	3R	Mitochondrial ribosomal protein L37	1.5	0.000	2.1	0.003	2.1	0.003	1.8	0.000	1.5	0.012
CG7726	2R	Ribosomal protein L11	1.5	0.001	1.5	0.043	1.9	0.003	1.8	0.001	1.8	0.000
CG1475	3R	Ribosomal protein L13A	1.3	0.017	1.5	0.119	2.3	0.003	2.0	0.000	1.8	0.000
CG3203	X	Ribosomal protein L17	1.2	0.061	1.3	0.064	2.0	0.002	1.8	0.000	1.6	0.000
CG7434	X	Ribosomal protein L22	1.3	0.001	1.2	0.174	1.8	0.003	1.6	0.003	1.5	0.000
CG4759	3R	Ribosomal protein L27	1.2	0.022	1.6	0.001	2.0	0.000	1.9	0.000	1.5	0.001
CG4111	X	Ribosomal protein L35	1.5	0.017	1.6	0.046	2.0	0.002	2.2	0.000	1.9	0.000
CG12324	2R	Ribosomal protein S15Ab	1.3	0.001	1.2	0.060	1.8	0.007	1.5	0.002	1.3	0.013
CG5920	2L	Ribosomal protein S2	1.3	0.003	1.3	0.040	2.1	0.001	1.9	0.000	1.6	0.000
CG8415	2R	Ribosomal protein S23	1.3	0.010	1.4	0.109	2.0	0.001	2.0	0.001	1.9	0.000
CG10423	3R	Ribosomal protein S27	1.5	0.003	1.8	0.081	2.6	0.004	2.4	0.000	2.3	0.000
CG2168	4	Ribosomal protein S3A	2.0	0.053	1.2	0.101	1.8	0.003	1.7	0.055	2.0	0.140
RE43488	2R	eIF-5A homolog, translational initiation factor activity	1.5	0.011	1.9	0.018	1.6	0.025	1.7	0.002	1.4	0.040

Table 5-5. (continued)

Gene name	Chromosome	Function and product	Day1		Day45		95%		90%		50%	
			fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.
Transport/ion binding												
GH06377	2R	AQP homolog. transporter activity	1.2	0.031	1.3	0.031	1.5	0.117	1.9	0.001	1.8	0.000
CG11739	3R	Cation transport. tricarboxylate carrier activity	1.2	0.043	1.9	0.075	1.4	0.024	1.2	0.006	1.4	0.000
CG2216	3R	Iron ion binding	1.2	0.018	1.6	0.031	1.8	0.004	1.7	0.002	1.3	0.014
CG5708	2L	Zinc ion binding	1.2	0.018	1.3	0.018	2.0	0.001	1.5	0.000	1.4	0.006
CG12794	X	Phosphate transport	1.2	0.007	1.7	0.004	1.5	0.028	1.3	0.035	1.3	0.046
CG32576	X	Vesicle-mediated transport	1.3	0.001	2.0	0.001	1.9	0.013	1.4	0.000	1.4	0.000
Transcriptional regulation												
tai	2L	Transcription coactivator activity, signal transducer activity	1.2	0.098	1.4	0.030	1.5	0.050	1.4	0.001	1.5	0.001
Mst77F	3L	Male-specific transcript 77F, sperm chromatin condensation	1.5	0.029	1.6	0.096	1.3	0.056	1.5	0.000	2.0	0.000
bic	2R	Transcription factor activity	1.1	0.302	1.3	0.169	1.8	0.002	1.0	0.425	1.3	0.420
CG11071	X	Transcription factor activity, zinc ion binding	1.3	0.004	1.4	0.181	1.9	0.006	1.6	0.002	1.6	0.002
Signaling/cell defense/others												
CG5333	3R	Apoptosis (Bjorklund 2006, Bolkan 2006)	1.3	0.004	1.3	0.105	1.8	0.003	1.5	0.003	1.7	0.003
CG31764	2L	Defense response to virus	1.2	0.021	1.3	0.049	1.5	0.000	1.7	0.003	1.2	0.017
Ste12DOR	X	Stellate 12D orphon, protein kinase CK2 regulator activity	1.0	0.804	3.3	0.091	1.3	0.051	1.3	0.025	1.3	0.002
CG2827	2R	Transaldolase activity	1.1	0.093	1.8	0.032	1.7	0.001	1.6	0.007	1.6	0.005
CG4609	3L	Transmission of nerve impulse	1.2	0.055	1.3	0.017	1.3	0.009	1.3	0.038	1.5	0.021
CG8893	X	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating) activity. Glycolysis	1.3	0.004	1.2	0.098	1.7	0.008	1.6	0.002	1.3	0.022
CG3989	X	Purine base metabolism. phosphoribosylaminoimidazole carboxylase activity	1.1	0.013	1.4	0.007	1.6	0.001	1.3	0.076	1.3	0.098

Table 5-5. (continued)

Gene name	Chromosome	Function and product	Day1		Day45		95%		90%		50%	
			fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.	fold	p-val.
CG10863	3L	Aldehyde reductase activity	1.3	0.053	2.4	0.095	1.4	0.015	1.2	0.038	1.2	0.064
CG3572	2R	Ral GTPase binding, intracellular signaling cascade	1.7	0.001	1.6	0.026	1.8	0.006	1.4	0.016	1.2	0.056
CG32672	X	Structural constituent of cytoskeleton	1.4	0.008	1.7	0.075	1.9	0.005	1.3	0.004	1.3	0.001
CG3532	3R	Structural constituent of cytoskeleton	1.5	0.007	1.5	0.007	1.7	0.003	1.9	0.001	1.6	0.016
CG6310	3L	Long-term memory (Dubnau 2003)	1.4	0.004	2.4	0.077	1.4	0.069	1.3	0.022	1.8	0.000
Unknown function												
CG15098	2R	Unknown	1.1	0.031	1.5	0.007	2.1	0.002	1.9	0.001	1.6	0.003
CG16826	2L	Unknown	1.4	0.018	1.3	0.025	2.5	0.002	1.9	0.005	2.0	0.001
CG2267	3R	unknown	1.2	0.068	1.5	0.008	1.4	0.012	1.4	0.003	1.4	0.000
CG40127	2R	unknown	1.3	0.001	1.4	0.011	1.8	0.001	1.7	0.026	1.3	0.060
CG9396	3R	unknown	1.1	0.006	1.5	0.014	1.9	0.004	1.3	0.000	1.4	0.005
CG8229	2R	unknown	1.3	0.001	1.5	0.016	2.3	0.130	1.4	0.005	1.3	0.000
CG14327	3R	unknown	1.3	0.001	1.4	0.009	1.4	0.031	1.4	0.078	1.4	0.011
CG31286	3R	unknown	1.3	0.070	1.4	0.071	1.3	0.007	1.3	0.017	1.5	0.006
CG14757	2R	unknown	1.4	0.005	3.2	0.000	1.5	0.013	1.8	0.000	1.7	0.000
GM03761	3R	unknown	1.3	0.039	1.3	0.030	1.4	0.012	1.5	0.000	1.1	0.058
CG10570	2L	unknown	1.3	0.069	2.1	0.002	1.9	0.007	1.3	0.029	1.2	0.033
CG5089	2R	unknown	1.3	0.006	1.4	0.095	1.3	0.072	1.2	0.097	1.5	0.000

5.5.6 How does Hsp22 increase lifespan?

The question remains on how Hsp22 exerts its beneficial effect on lifespan. Our microarray experiments suggest that this beneficial effect involves mitochondrial function, protein biosynthesis, proteolysis and cell defense. From these functions two pictures can be drawn on the effect of Hsp22 over-expression in *Drosophila*.

A normal aging is characterized by a decrease in mRNA transcripts accompanied by a consistent decrease of the rate of protein synthesis and a decline of the rate of total protein turnover (Rattan, 1996; Calleja et al., 1993; Bailey and Webster, 1984; Fleming et al., 1986). Taking into account that the total number of polypeptides remains constant with age, a reduced turnover rate would imply that polypeptides remain longer in the cell before being replaced. If an enzyme is more susceptible to post-translational alterations due to oxidative damage, then the functional capacity of the mitochondria and of the cell would be affected because of the greater proportion of old enzymes.

Hsp22+ flies display an increase of genes involved in protein biosynthesis and proteolysis suggesting a better turnover of proteins than in control flies. As part of the cell defense system, ROS scavenging enzymes and Hsp22 by-itself could also contribute to a decrease in the amount of misfolded/damaged proteins in the whole cells and particularly in mitochondria. Therefore, Hsp22+ flies would accumulate less damaged proteins and keep a better balance in their cellular and mitochondrial homeostasis. Arguing in this sense, genes involved in the electron transport chain, which have been suggested to be aging biomarkers by Curtis et al. (2007), are up-regulated in Hsp22+ flies.

Alternatively, it can be hypothesized that Hsp22 over-expressing flies have a higher energy production than control flies from the consistent up-regulation of genes involved in oxidative phosphorylation. However, more energy production could lead to increased ROS generation and increased damages to proteins or molecules in the cell, which would account for the up-regulation of genes related to protein turnover such as protein folding and proteolysis to remove damaged protein. Thus, the ATP-dependent proteolysis process would again need energy. At the same time, the protein synthesis process would be activated to replace damaged proteins, and lipid metabolism would be involved in signaling pathway or supporting cell homeostasis. This hypothesis is schematically summarized in Fig. 5-9.

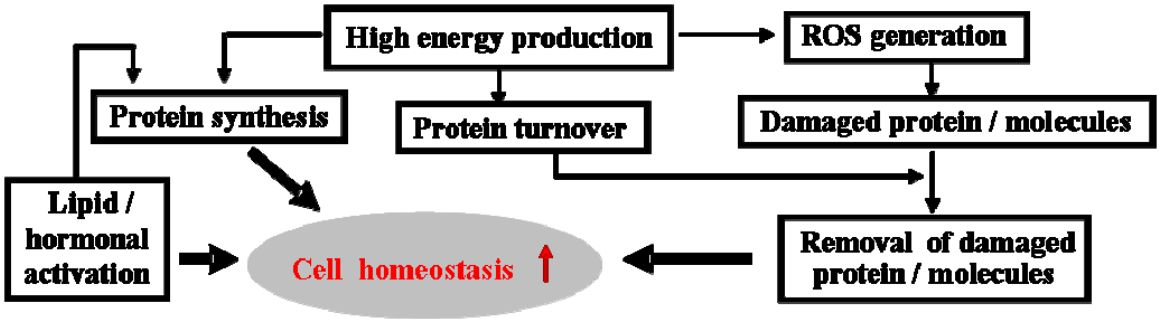

Fig. 5-9. Putative relationship between functional categories mainly up-regulated in Hsp22+ flies

In summary, long-lived Hsp22+ flies have a higher transcriptional level of genes involved in mitochondrial function, protein biosynthesis, proteolysis and cell defense which are mostly down-regulated during normal aging. The positive regulation of these genes in Hsp22+ flies could be advantageous by favoring/keeping a good cellular and mitochondrial homeostasis.

5.6 Acknowledgements

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5.7 Appendix: Additional data files

Western blot showing Hsp22 over-expression by UAS/GAL4 system.
(Additional data file A)

Down-regulated genes at day 1
(Additional data file B)

Down-regulated genes at day 45
(Additional data file C)

Down-regulated genes at 95% survival
(Additional data file D)

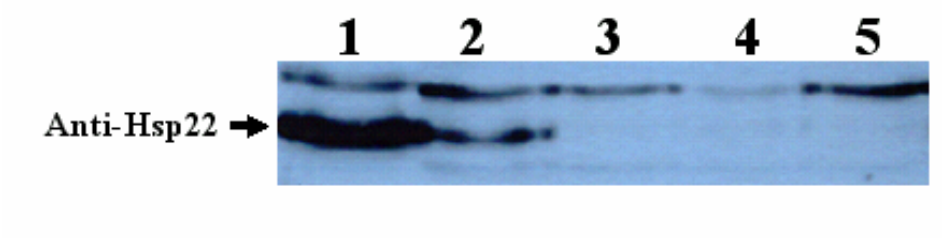
Down-regulated genes at 90% survival
(Additional data file E)

Down-regulated genes at 50% survival
(Additional data file F)

Common genes of down-regulation
(Additional data file G)

Age-dependent expression of antioxidant activity genes
(Additional data file H)

5.7.1 Western blot showing Hsp22 over-expression by UAS/GAL4 system (Additional data file A)



1. Heat shocked *Drosophila* S2 cells

2. *actin*-Gal4;EP(3)3247 flies (Hsp22+), 7days-old

3. *cyo*;EP(3)3247 (control 1), 7 days-old

4. *actin*-Gal4;TM3,*sb* (control 2), 7 days-old

5. *cyo*;TM3,*sb* (control), 7 days-old

Siblings (7-day-old) obtained from the crossing between *actin-GAL4/cyo* male flies and *EP(3)3247/TM3sb* female flies were used for protein analysis. Protein extracts from flies were separated on 12% SDS-PAGE. The SDS polyacrylamide gels were prepared as outlined by Thomas and Kornberg (1975), with modification in the pH of the running buffer (8.5 instead of 8.8) and in the acrylamide:bis ratio (30:0.8 instead of 30:0.15). These conditions resulted in a better resolution for the sHsps. Following transfer on nitrocellulose membranes, Western blot was performed using polyclonal rabbit antibody against Hsp22 (#36, 1/2500; Morrow et al., 2000). Peroxidase-conjugated secondary antibody of goat anti-rabbit (1/10,000, Jackson ImmunoResearch laboratories, West Grove, PA) were used. Chemiluminescent detection was done using Western Lightning Chemiluminescence Reagent (PerkinElmer Life Sciences, Boston, MA) according to the manufacturer's instruction.

5.7.2 Down-regulated genes at day 1 (Additional data file B)

Additional data file B.

Down-regulated genes at day 1

Spot No.	Gene ID	Gene mean (Log 2 ratio)	p value	Gene annotation
8231	*CG18522*	-1,8520902	0,00461711	oxidoreductase activity
1775	*CG32484*	-1,6427789	0,007049584	carbohydrate kinase activity
25346	*CK00026*	-1,4441472	0,002291144	unknown
23296	*CG7462*	-1,4053441	0,006349879	receptor binding, structural constituent of cytoskeleton
25345	*CK00026*	-1,3848162	0,002464933	unknown
25226	*GH07363*	-1,3064401	0,008012673	unknown
19193	*CG7642*	-1,224863	8,32E-04	oxygen and reactive oxygen species metabolism, xanthine dehydrogenase activity
1959	*CG1106*	-1,1929687	0,00395758	structural constituent of cytoskeleton
26529	*CG11081*	-1,1917582	6,13E-05	transmembrane receptor protein tyrosine kinase activity
27405	*CG13737*	-1,1776047	0,002064243	unknown
50	*CG8915*	-1,163154	7,82E-05	ATP-dependent RNA helicase activity
25160	*CG4250*	-1,1122698	0,001459529	unknown
26011	*CG11842*	-1,0574735	2,72E-04	serine-type endopeptidase activity
49	*CG8915*	-1,0533623	6,25E-05	ATP-dependent RNA helicase activity
732	*CG1031*	-1,0353812	0,006909669	unknown
23687	*CG1692*	-1,0280113	0,003816646	Mo-molybdopterin cofactor sulfurase activity
18506	*CG1945*	-1,0217167	0,005395961	ubiquitin-specific protease activity, protein deubiquitination
24959	*CG8345*	-1,016468	0,009348773	oxidoreductase activity, electron transporter activity
24371	*CG30147*	-1,0039049	0,002821222	unknown

5.7.3 Down-regulated genes at day 45 (Additional data file C)

Additional data file C.

Down-regulated genes at day 45

Spot No.	Gene ID	mean (Log 2	p value	Gene annotation
19389	CG1915	-3,3424523	0,002319197	structural constituent of cytoskeleton
5437	CG2999	-3,111117	0,009097385	receptor activity, calmodulin binding
14783	RH25742	-2,918302	0,001455866	unknown
6235	CG3290	-2,1287308	0,009732791	alkaline phosphatase activity
17202	SD27348	-2,1274316	0,002474955	unknown
4240	CG8223	-2,0450037	6,30E-05	unknown
14784	RH25742	-1,9978666	0,008399696	unknown
13455	CG1658	-1,9487761	0,006223826	unknown
6236	CG3290	-1,9250215	0,002816826	alkaline phosphatase activity
18063	CG10435	-1,8851674	0,004224971	unknown
10742	RH07382	-1,8811787	0,005508771	unknown
27232	CG2903	-1,8120824	0,009902839	protein binding
4236	CG5384	-1,7888571	0,00120357	ubiquitin-specific protease activity
3087	CG12505	-1,7634048	0,003022386	unknown
4239	CG8223	-1,7411146	7,83E-04	unknown
5432	CG32477	-1,6985054	0,006263023	unknown
4235	CG5384	-1,690023	7,97E-04	ubiquitin-specific protease activity
5431	CG32477	-1,6770767	0,003946154	unknown
10106	CG12156	-1,6634593	0,006067424	phagocytosis, lysosomal transport, GTPase activity
3088	CG12505	-1,6295855	0,006094958	unknown
21426	CG4589	-1,6257083	0,008707431	calcium ion binding
14782	CG7378	-1,606322	0,003829484	protein tyrosine/serine/threonine phosphatase activity
23200	CG5383	-1,6006593	0,005360909	unknown
3083	CG8032	-1,5772518	0,003388879	oxidoreductase activity
18064	CG10435	-1,551932	0,005545813	unknown
2379	CG10888	-1,530695	0,009530009	G-protein coupled photoreceptor activity
3659	CG8603	-1,4840157	0,003855896	ion transport
13456	CG1658	-1,446695	0,007048402	unknown
3084	CG8032	-1,4457772	0,006815489	oxidoreductase activity
4815	CG10724	-1,4294791	0,009624139	structural constituent of cytoskeleton
20550	CG31847	-1,4221802	0,001499718	unknown
13484	SD02026	-1,3858502	0,003489887	unknown
7635	CG15669	-1,3659669	0,003679043	unknown
2377	CG31683	-1,352203	0,002234072	phospholipase activity, hydrolase activity
2536	CG6686	-1,3506455	0,002005826	transcription regulator activity
3103	CG13124	-1,3472456	5,64E-04	unknown
1954	CG15609	-1,331637	0,005394477	structural constituent of cytoskeleton
23645	CG17265	-1,3276739	0,002775356	unknown
17510	CG12876	-1,2925258	0,007397577	receptor signaling protein activity, induction of apoptosis
8848	CG1105	-1,278796	0,008046933	unknown
16521	CG9668	-1,2618576	0,001000028	G-protein coupled photoreceptor activity
7050	CG17739	-1,2600148	0,005378204	structural molecule activity
14176	CG10261	-1,2385607	9,25E-04	receptor signaling protein serine/threonine kinase activity
8020	CG10045	-1,2169461	0,001229098	glutathione transferase activity, defense response
21556	CG18102	-1,2105417	0,004096439	unknown
24367	CG6461	-1,1880856	4,42E-05	peptidase activity, acyltransferase activity

Additional data file C (continued).

Spot No.	Gene ID	mean (Log 2	p value	Gene annotation
25944	CG17100	-1,1775281	0,003394423	RNA polymerase II transcription factor activity
26762	SD10530	-1,172109	0,006732594	unknown
25943	CG17100	-1,1567339	0,005860597	RNA polymerase II transcription factor activity
4255	CG15095	-1,1523995	0,007157865	sodium symporter activity
9503	CG31272	-1,1258482	0,001002573	transporter activity
18570	GH10215	-1,1039605	0,007413442	unknown
26761	SD10530	-1,0967247	0,003696151	unknown
3634	CG6355	-1,0832119	0,002069603	zinc ion binding
11589	CG5061	-1,0794078	0,006355438	structural constituent of cytoskeleton
5939	CG31332	-1,0611818	8,10E-04	structural constituent of cytoskeleton
24368	CG6461	-1,0596926	0,001311362	acyltransferase activity
9504	CG31272	-1,0389537	0,003788017	transporter activity
24341	CG32688	-1,0358713	7,95E-04	voltage-gated potassium channel activity
4836	CG18783	-1,0077652	0,006933668	transcription factor activity

5.7.4 Down-regulated genes at 95% survival (Additional data file D)

Additional data file D.

Down-regulated genes at 95% survival

Spot No.	Gene ID	mean (Log 2	p value	Gene annotation
5605	CG13870	-1,7347827	0,008228318	unknown
3069	CG3616	-1,6226677	0,004762661	oxidoreductase activity
25543	CG2854	-1,5904998	6,89E-05	unknown
1189	HL03650	-1,4597343	0,002775696	unknown
23453	RH68619	-1,4028397	0,00987231	unknown
25544	CG2854	-1,354518	0,003211489	unknown
24253	CG1744	-1,3320704	0,005828918	receptor activity, structural molecule activity
14402	CG32019	-1,277238	0,005758722	receptor signaling protein serine/threonine kinase activity
15764	CG14355	-1,2630363	0,007259774	unknown
8109	CG6091	-1,2539504	2,08E-05	unknown
1015	CG8219	-1,2175661	6,43E-04	protein-nucleus import, protein carrier activity
19621	CG9368	-1,1967329	0,004692115	unknown
22915	CG5794	-1,1570609	0,004457528	cysteine-type peptidase activity
10007	CG31025	-1,1485426	0,003733356	unknown
14291	CG18345	-1,1224346	0,004415761	calcium channel activity
7363	CG3222	-1,114224	0,006625814	unknown
25510	CG4900	-1,1095709	0,002475317	mRNA binding, iron ion binding
2783	CG1824	-1,0970209	7,18E-04	xenobiotic-transporting ATPase activity
12318	CG32940	-1,091015	0,004658246	unknown
16927	CG3920	-1,0888367	0,004873348	unknown
15166	CG5105	-1,0877024	0,005437299	phospholipase A2 activator activity
16586	CG13337	-1,0842623	0,008705861	unknown
6526	CG32026	-1,0443531	0,005068861	oxidoreductase activity, isocitrate dehydrogenase (NAD+) activity
17458	CG9701	-1,0426637	3,38E-04	glucosidase activity, hydrolase activity
21761	CG17268	-1,0341508	0,009761162	unknown
3633	CG6355	-1,0274373	0,002262629	zinc ion binding
9236	LD05680	-1,0270592	0,005014238	unknown
22078	CG5092	-1,0249621	0,008135759	protein kinase activity
3634	CG6355	-1,0145694	0,00606481	zinc ion binding
12475	RH14292	-1,0122353	0,003101342	unknown

5.7.5 Down-regulated genes at 90% survival (Additional data file E)

Additioanl data file E.

Down-regulated genes at 90% survival

Spot No.	Gene ID	mean (Log 2	p value	Gene annotation
301	RE18078	-1,9057509	1,69E-05	unknown
13950	CG4847	-1,738387	0,00689977	proteolysis and peptidolysis, cathepsin K activity
13484	SD02026	-1,5368767	9,47E-06	unknown
13483	SD02026	-1,4285679	2,50E-04	unknown
13047	RH03309	-1,1295925	0,009986697	unknown
13	CG5224	-1,1059597	0,003286581	defense response, glutathione transferase activity
5471	CG10924	-1,0647808	0,00312128	phosphoenolpyruvate carboxykinase (GTP) activity
5472	CG10924	-1,0497864	0,003422475	phosphoenolpyruvate carboxykinase (GTP) activity
5641	CG11201	-1,010904	0,005795415	structural constituent of cytoskeleton

5.7.6 Down-regulated genes at 50% survival (Additional data file F)

Additional data file F.

Down-regulated genes at 50% survival

Spot No.	Gene ID	mean (Log 2	p value	Gene annotation
26529	CG11081	-2,5050166	0,00818255	transmembrane receptor protein tyrosine kinase activity
23975	CG1291	-1,9555794	0,006595342	glycolipid mannosyltransferase activity
14402	CG32019	-1,9459218	0,001769457	receptor signaling protein serine/threonine kinase activity
13852	CG30438	-1,9355823	0,002170784	transferase activity, transferring glycosyl groups
9618	CG1915	-1,7604519	0,002857073	structural constituent of cytoskeleton
9617	CG1915	-1,7244545	0,001588784	structural constituent of cytoskeleton
11840	CG31607	-1,6811317	0,00527858	unknown
19194	CG7642	-1,6638373	0,003978581	oxygen/reactive oxygen species metabolism, xanthine dehydrogenase activity
26696	CG7365	-1,5225462	0,001143564	phospholipase activity
19645	CG11111	-1,5051961	0,007342458	phosphatidylinositol transporter activity
2711	CG3504	-1,4905901	0,007505555	structural molecule activity, calmodulin binding,
13851	CG30438	-1,4613472	0,005537855	transferase activity, transferring glycosyl groups
2227	LD17738	-1,4383553	0,003764919	unknown
11120	CG32475	-1,4261074	0,007945331	G-protein coupled receptor activity
14292	CG18345	-1,3292922	2,20E-04	calcium channel activity, calmodulin binding
7559	CG4389	-1,3238713	0,003926727	long-chain-enoyl-CoA hydratase activity
21556	CG18102	-1,3223354	0,003568729	unknown
7707	CG1486	-1,2948881	0,008610822	unknown
22935	CG11671	-1,2806972	0,002901404	unknown
14291	CG18345	-1,2797589	0,001105816	calcium channel activity, calmodulin binding
12915	CG3359	-1,2573017	0,002146162	signal transduction
13484	SD02026	-1,209792	7,53E-05	unknown
9911	CG30118	-1,1857543	2,42E-04	unknown
7164	CG3725	-1,1831046	0,002284617	calcium-transporting ATPase activity
4669	CG7324	-1,179516	0,005879351	Rab GTPase activator activity
22988	CG18234	-1,1789856	0,007676046	oxidoreductase activity
16840	CG5455	-1,1658765	0,009397158	unknown
5939	CG31332	-1,1649734	5,50E-04	structural constituent of cytoskeleton
21555	CG18102	-1,1636404	4,16E-04	unknown
4745	CG32626	-1,1593524	0,008055385	AMP deaminase activity
14302	CG9451	-1,1586742	0,001448696	acid phosphatase activity
17733	CG32477	-1,1256055	3,44E-04	unknown
27205	CG7507	-1,1161937	0,006317879	microtubule motor activity,structural constituent of cytoskeleton
11552	CG15117	-1,1151464	6,51E-04	beta-glucuronidase activity
15223	CG8095	-1,1063486	0,004139845	receptor activity
2061	CG30438	-1,1042526	0,001266322	transferase activity, transferring glycosyl groups
24253	CG1744	-1,0728443	1,81E-04	receptor activity, structural molecule activity
18477	CG6327	-1,0646911	0,004383225	unknown
18097	CG6967	-1,0612584	0,004643654	nucleic acid binding, DNA helicase activity
14994	CG6805	-1,0592088	0,005937197	inositol trisphosphate phosphatase activity
21849	CG15102	-1,0438861	0,009226336	epoxide hydrolase activity, juvenile hormone epoxide hydrolase activity
27190	CG9057	-1,0386786	4,48E-04	lipid storage
7163	CG3725	-1,0375123	0,003069282	calcium-transporting ATPase activity
25376	CG17678	-1,0349721	0,004885969	unknown
12929	CG9012	-1,0325269	6,92E-04	intracellular protein transport
13903	CG5518	-1,0243454	7,47E-04	proteolysis and peptidolysis, membrane alanyl aminopeptidase activity
25943	CG17100	-1,0009453	0,001610393	RNA polymerase II transcription factor activity

5.7.7 Common genes of down-regulation (Additional data file G)

Additional data file G.

1) Common down-regulation in chronological ages

Spot No.	Gene ID	Gene name	Day 1		Day 45		Gene annotation
			Fold (Log2)	p value	Fold (Log2)	p value	
1250	*CG10146*	*Attacin-A*	-1,235	0,0216	-1,005	0,3420	defense response to Gram-negative bacteria
2952	*CG3051*	*SNF1A*	-1,072	0,0764	-1,270	0,0178	protein serine/threonine kinase activity
4759	*CG2151*	*Thioredoxin reductase-1*	-1,154	0,0187	-2,314	0,1750	thioredoxin-disulfide reductase activity, determination of adult life span
5404	*CG30115*	*CG30115*	-1,026	0,0279	-1,156	0,1247	guanyl-nucleotide exchange factor activity
18505	*CG1945*	*faf*	-1,112	0,0235	-1,096	0,0553	ubiquitin-specific protease activity, negative regulation of proteolysis and peptidolysis
18506	*CG1945*	*faf*	-1,022	0,0054	-1,406	0,0729	ubiquitin-specific protease activity, negative regulation of proteolysis and peptidolysis

2) Common down-regulation in physiological ages

No detected

3) Common down-regulation in 50% survival/45 days-old

Spot No.	Gene ID	Gene name	50% survival		Day 45		Gene annotation
			Fold (Log2)	p value	Fold (Log2)	p value	
5939	*CG31332*	*unc-115*	-1,165	0,0005	-1,061	0,0008	structural constituent of cytoskeleton
9617	*CG1915*	*sallimus*	-1,724	0,0016	-3,342	0,0023	structural constituent of cytoskeleton
9618	*CG1915*	*sallimus*	-1,760	0,0029	-3,342	0,0023	structural constituent of cytoskeleton
13484	*SD02026*	-	-1,210	0,0001	-1,386	0,0035	unknown
17733	*CG32477*	*CG32478*	-1,126	0,0003	-1,699	0,0063	unknown
21555	*CG18102*	*shibire*	-1,164	0,0004	-1,211	0,0041	unknown
21556	*CG18102*	*shibire*	-1,322	0,0036	-1,211	0,0041	synaptic vesicle endocytosis, microtubule motor activity, GTPase activity
25943	*CG17100*	*CG17101*	-1,001	0,0016	-1,178	0,0034	RNA polymerase II transcription factor activity

5.7.8 Age-dependent expression of antioxidant activity genes (Additional data file H)

Additional data file H.

Antioxidant activity genes

Day 1	Spot No.	ID	Gene name	Gene ratio	Raw p value
	15415	CG6888	*thioredoxin peroxidase activity*	2,1	0,012516805
	5255	CG8938	*Glutathione S transferase S1*	1,9	0,019931853
	5256	CG8938	*Glutathione S transferase S1*	1,7	0,000947002

Day 45	Spot No.	ID	Gene name	Gene ratio	Raw p value
	5255	CG8938	*Glutathione S transferase S1*	2,3	0,019305184
	5256	CG8938	*Glutathione S transferase S1*	1,9	0,002606954
	23686	CG1633	*thioredoxin peroxidase 1*	1,7	0,03658804
	23685	CG1633	*thioredoxin peroxidase 1*	1,7	0,024347842
	3538	CG8905	*sod2*	1,3	0,1614121

95% survival	Spot No.	ID	Gene name	Gene ratio	Raw p value
	20367	CG12013	*glutathione peroxidases*	1,6	0,020244084
	23686	CG1633	*thioredoxin peroxidase 1*	1,5	0,00611415
	23685	CG1633	*thioredoxin peroxidase 1*	1,5	0,018836768
	20368	CG12013	*glutathione peroxidases*	1,5	0,007867143

90% survival	Spot No.	ID	Gene name	Gene ratio	Raw p value
	5255	CG8938	*Glutathione S transferase S1*	1,5	1,58678E-06
	20367	CG12013	*glutathione peroxidases*	1,4	0,005024577
	20368	CG12013	*glutathione peroxidases*	1,4	0,006730647
	23685	CG1633	*thioredoxin peroxidase 1*	1,4	0,006468286
	5256	CG8938	*Glutathione S transferase S1*	1,4	0,000579765
	23686	CG1633	*thioredoxin peroxidase 1*	1,3	0,008201538
	3538	CG8905	*sod2*	1,2	0,015535595
	3537	CG8905	*sod2*	1,2	0,000497826

50% survival	Spot No.	ID	Gene name	Gene ratio	Raw p value
	5255	CG8938	*Glutathione S transferase S1*	1,7	1,00281E-05
	5256	CG8938	*Glutathione S transferase S1*	1,6	8,47421E-05
	3538	CG8905	*sod2*	1,4	0,002945409
	20368	CG12013	*glutathione peroxidases*	1,4	0,012696081
	23685	CG1633	*thioredoxin peroxidase 1*	1,3	0,006351959
	20367	CG12013	*glutathione peroxidases*	1,3	0,026355801
	15109	CG9314	*catalase activity*	1,3	0,06738962
	2050	CG11793	*sod1*	1,2	0,031846214
	3537	CG8905	*sod2*	1,2	0,00045625

Chapter 6. General discussion and conclusion

The aim of this work was to understand the mechanisms behind Hsp22-associated longevity by defining the differential proteomic and transcriptomic profiles in the long-lived flies over-expressing Hsp22. The Hsp22 protein has *in vitro* chaperone-like activity (Morrow et al., 2006) and has been localized in the mitochondrial matrix in *Drosophila*. Therefore, the function of Hsp22 in the aging process is likely related to mitochondria. In Harman' free radical theory on aging, it was proposed that the aging process as well as the aging-related degenerative diseases are caused by the deleterious effects of free radicals on various cell components (Harman, 1956). A close relationship between the generation of free radicals and the metabolic rate and the mitochondrial respiration has been hypothesized. Thus, a balance between the intracellular accumulation of ROS and the activity of ROS scavenging enzymes seems to be a significant factor influencing the aging process. The relationship between oxygen consumption and ROS generation however, seems to be more complex than generally predicted and several relevant intracellular targets of ROS have recently been detected (Finkel and Holbrook, 2000; Nemoto et al., 2000; Nishikawa et al., 2000).

A comparison of genes and proteins which show similar trends in transcriptome and in proteome is summarized in Table 6-1. Some gene products come out in both analysis while others do not. Among the former, the genes and proteins of ATP synthase subunit beta NADH dehydrogenase (mitochondrial electron transport chain enzyme), 3-hydroxyisobutyrate dehydrogenase (amino acid metabolism), (L)-malate dehydrogenase (TCA cycle, glycolysis), enoyl-CoA hydratase (fatty acid beta-oxidation), Glutathione S-transferase S1 (antioxidant activity) and Tubulin beta-2 chain showed concomitent increase in their expressions in Hsp22 over-expressing flies. Taken together with apparently increased functions obtained in proteomic/transcriptomic analysis and additional observation on physiological features, the obtained results indicate several specificities in the long-lived flies over-expressing Hsp22.

Table 6-1. Comparison of expression of genes and proteins observed in transcriptome and in proteome

Protein	Gene name	Proteome (Hsp22+ / common)	Transcriptome (Hsp22+ / controls)				
			Day 1	Day 45	95% survival	90% survival	50% survival
ATP synthase subunit beta, mitochondrial precursor	*CG11154*	Up (1.31-1.32)	1.5 - 1.6	1.8	1.6 - 1.8	1.6 - 1.7	1.4
NADH dehydrogenase (ubiquinone), mitochondrial respiratory chain complex I	*CG12079*	Up (1.55)	1.0 - 1.1	1.8	1.1 - 1.6	1.2 - 1.3	1.3 - 1.4
Probable medium-chain specific acyl-CoA dehydrogenase, mitochondrial precursor	*CG12262*	Up (1.47)	0.9 - 1.1	0.4 - 1.6	1.4 - 1.5	0.8 - 1.0	0.6 - 0.8
Probable 3-hydroxyisobutyrate dehydrogenase, mitochondrial precursor	*CG15093*	Up (1.34)	1.1	1.6 - 2.0	1.5	1.1	1.6
Cathepsin D, proteolysis (eukaryotic aspartyl protease)	*CG1548*	Up (1.87)	NE[a]	NE	NE	NE	NE
NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	*CG2286*	Up (1.53-1.60)	ND[b]	ND	ND	ND	ND
Alcohol dehydrogenase	*CG3481*	Up (1.54)	0.9 - 1.0	ND	ND	ND	ND
Vacuolar ATP synthase catalytic subunit A isoform 2	*CG3762*	Up (1.28)	0.9	0.8 - 1.0	0.8 - 0.9	0.9	0.8
Isocitrate dehydrogenase (oxidoreductase), TCA cycle	*CG5028*	Up (1.43)	NE	NE	NE	NE	NE
Malate dehydrogenase, TCA cycle	*CG5889*	Up (1.57)	1.0 -1.1	2.2 - 2.4	1.0 - 1.3	1.0	1.2
Mitochondrial glycoprotein, Q subcomponent binding protein	*CG6459*	Up (1.35)	0.8	0.8 - 1.0	0.8 - 1.0	0.9	0.9

Table 6-1. (continued)

Protein	Gene name	Proteome (Hsp22+ / common)	Transcriptome (Hsp22+ / controls)				
			Day 1	Day 45	95% survival	90% survival	50% survival
Enoyl-CoA hydratase (oxidoreductation, fatty acid beta-oxidation)	*CG6543*	Up (1.51)	1.0 -1.2	1.7 - 1.9	1.1 - 1.3	1.2 - 1.3	1.2 - 1.4
Glutamate dehydrogenase, mitochondrial precursor	*CG5320*	Up (1.44-1.62)	1.0 - 1.1	0.7 - 0.8	0.6 - 0.9	1.0	1.0
Voltage-dependent anion-selective channel (porin, DmVDAC)	*CG6647*	Up (1.55)	0.9 - 1.0	0.9	1.1 - 1.2	1.0	1.1
L-malate dehydrogenase (L-lactate dehydrogenase), TCA cycle, glycolysis	*CG7998*	Up (1.57-1.69) Down (0.47-0.78)	1.1 - 1.2	1.0 - 1.4	1.3 - 1.4	0.9 - 1.0	1.1 - 1.2
Glutathione S-transferase S1	*CG8938*	Up (1.45)	1.7 - 1.9	1.9 - 2.3	0.7	1.4 - 1.5	1.6 - 1.7
Tubulin beta-2 chain	*CG9359*	Up (1.72)	1.3	1.0	1.2	1.4	1.4 - 1.5
Actin-87E	*CG18290*	Down (0.69)	0.9 - 1.1	0.7 - 0.8	0.9 - 1-1	1.0 - 1.1	1.0 - 1.3

[a]NE; not existing gene on the microarray chip used in this experiment
[b]ND; not detected gene by low spot intensity or non-hybridization during microarray

6.1 Hsp22 over-expressing flies have an increased oxidative stress resistance

The long-lived flies over-expressing Hsp22 had an increased capacity to survive under the oxidative stress caused by paraquat. Under normal cellular condition, the major production of ROS is generated through the mitochondrial respiratory chain (Ambrosio et al., 1993). 0.4-4% of the total oxygen consumed by mitochondrion is estimated to be converted into ROS (Giulivi et al., 1995; Gudz et al., 1997). Natural antioxidant mechanisms including a manganese-dependent superoxide dismutase (MnSOD) exist within the mitochondrion to limit oxidative damage (Fridovich, 1995; Green et al., 2004). Additional forms of SOD exist in the cytoplasm or extracellular space to scavenge ROS outside the mitochondrion. Chen et al. (2003) reported that the released ROS from complex I (NADH dehydrogenase site) and complex III (Qi site) are directed toward matrix antioxidant defenses such as, manganese superoxide dismutase (MnSOD), glutathione (GSH), glutathione peroxidase (GPX), and glutathione reductase (GRase). However, the Qo site of complex III releases the ROS toward the intermembrane space, away from matrix antioxidant defenses, favoring release from mitochondria (Chen et al., 2003). Therefore, it was suggested that ROS production and release, and the site of the free radical damages to the cell might differ depending on the location of mitochondrial inhibition (Sedensky and Morgan, 2006).

Besides the deleterious effect of ROS, several evidences implicate ROS as specific signaling molecules under both physiological and pathophysiological conditions and as essential factors to maintain homeostasis in biological processes (Finkel and Holbrook, 2000). For example, ROS generation by phagocytic cells constitutes an essential host defense mechanism necessary to combat infection, or cytosolic ROS production in response to stimulation by growth factors are involved in regulating the proliferative response (Finkel, 1998). Although chronic ROS production may have deleterious effect, mitochondrial oxidants can also function acutely as signaling molecules to provide communication between the mitochondria and the cytosol by activating a novel metabolic regulatory pathway (Nemoto et al., 2000).

Interestingly, the Hsp22 over-expressing flies displayed increased expression of proteins and genes related to antioxidant function in addition to paraquat resistance. The expression of several antioxidant genes of the Hsp22+ flies vs. the control flies is summarized in Table 6-2.

In crude mitochondria, Hsp22+ flies also showed 1.45-fold (59.2% in Hsp22+ file vs. 40.8% in control) increased abundance of 'Glutathione S-transferase S1' protein. From the observation both at the transcript level (Chapter 5) and in the mitochondrial proteome (Chapter 4), Hsp22 over-expressing flies have preferentially increased expression of 'Glutathione S-transferase S1'.

Table 6-2. Antioxidant gene expression obtained from the transcriptomic analysis

Young (day1)	Old (day45)	95% survival	90% survival	50% survival
GstS1 (1.9)	*GstS1 (2.3)*	*GstS1 (0.7)*	*GstS1 (1.5)*	*GstS1 (1.7)*
		Glutathione peroxidase (1.6)	*Glutathione peroxidase (1.4)*	*Glutathione peroxidase (1.4)*
Trx peroxidase activity (2.1)				
	Trx peroxidase 1 (1.7)	*Trx peroxidase 1 (1.5)*	*Trx peroxidase 1 (1.4)*	*Trx peroxidase 1 (1.3)*
	Sod2 (1.3)		*Sod2 (1.2)*	*Sod2 (1.4)*
				Sod1 (1.2)
				Catalase activity (1.3)

The number indicated in parenthesis means relative ratio (Hsp22+ flies/control flies) of transcriptional gene expression.

Functional association between Hsp22 and Glutathione S-transferase S1 could be possible at their gene or protein level. Redox-sensitive genes or proteins and their regulatory factors have been implicated in similar cases of chaperone (or its regulatory) proteins. For instance, HSF stimulated by H_2O_2 in human endothelial cells increases its DNA binding activity and proceeds Hsps production (Jornot et al., 1997). Hsp70 stimulated by N-acetylcysteine reduces its own induction (Abe et al., 1998), while Hsp70 increases or alters mRNA induction by xanthine/xanthine oxidase or by SOD-1 (Yamamoto et al., 1993; Kamii et al., 1994; Kondo et al., 1997). AlphaB-crystallin, a member of the hsp27 family, is phosphorylated after treatment with H_2O_2 via activation of p38 and ERK MAP kinases (Ito et al., 1997). Once activated, the protein decreases ROS generation and stimulates GSH synthesis; this, in turn, results in diminished activation of NF-κB by oxidants (Mehlen et al., 1996).

Taken together, these results showing increased expression in antioxidant genes and protein may explain the increased resistance against paraquat oxidative stress in the Hsp22 over-expressing flies. Antioxidant defenses may be involved in the mechanism of longevity induced by Hsp22 over-expression.

6.2 Hsp22 over-expressing flies display a decrease of damaged proteins during lifespan

In addition to an increase in antioxidant activity, the Hsp22 over-expressing flies actually demonstrated an improved capacity to regulate oxidized proteins in aged flies as shown by a decreased accumulation of damaged proteins (Fig. 2-6). Protein oxidation resulting from a reaction with reactive oxygen (or nitrogen) species which are produced by intracellular aerobic metabolism at the peroxisome and the mitochondria (Beckman and Ames, 1998) are targeted to both amino acid side chains and peptidic backbone (Berlett and Stadtman, 1997). Oxidative damage to proteins can virtually affect all amino acids including sulfur-containing amino acids and aromatic amino acids (tyrosine, tryptophan or phenylalanine) being mainly susceptible to oxidation, and thus result in a wide variety of modified proteins and damaged products (Berlett and Stadtman, 1997). The most frequently, hydroxylated or carbonylated amino acid derivatives are irreversibly oxidized by changing their secondary or tertiary structures and forming protein-protein covalent cross-link or non-covalent aggregates (Grune et al., 1997; Bader and Grune, 2006). During the process of covalent cross-linking of protein aggregates, non-protein components such as carbohydrates and oxidized lipids can react with protein adducts and thus can increase the mass of oxidized aggregates (Berlett and Stadtman, 1997; Bader and Grune, 2006). Ultimately, they are directed for degradation preferentially by the 20S proteasome in an ATP- and ubiquitin-independent manner or by the ubiquitin-26S proteasome pathway, because these protein aggregates are poor substrates for proteases, and can accumulate within cells (Davies, 2001; Bader and Grune, 2006).

Perhaps, the induction of Hsp22 during normal aging is a response to oxidative stress as shown in the paraquat treatment (Fig. 2-9). Therefore, the over-expression of Hsp22 from the early age might contribute to the regulation of protein oxidation from the beginning of lifespan. However to our surprise, the Hsp22 over-expressing flies had a higher abundance of oxidized proteins than the control flies at the early age, and then showed gradually declining protein oxidation during aging. Thus, more complicated mechanisms seem to be involved in the longevity process in the Hsp22 over-expressing flies during lifespan.

The function of chaperone-like activity of Hsp22 may regulate damaged protein. The *in vitro* chaperone activity of Hsp22 compared to other small heat shock proteins is high as shown in Fig. 1-8. Unbalanced chaperone requirement and chaperone capacity in aged organisms cause

the accumulation of aggregated proteins, which often result in folding diseases, mostly in the nervous system, due to the limited proliferation of neurons. Therefore, over-expression of chaperones often delays the onset or diminishes the symptoms of the disease (Sőti and Csermely, 2002a and b), and increased chaperone induction leads to increased longevity (Tatar et al., 1997). Irreversibly damaged proteins are recognized by chaperones, and targeted for site-specific cellular degradation such as by proteolysis in the mitochondria, by proteasome system in the cytosol or by autophagy.

6.3 Hsp22 over-expressing flies have an enhanced activity of protein turnover

Under certain stress-conditions caused by the aging process, there is an accumulation of misfolded and aggregated proteins. This is partially due to post-translational modification by ROS. The cell has developed a number of antioxidant defense systems including superoxide dismutase, the peroxidases, and the glutathione redox cycle. However, the activity of these enzymes decreases during aging, which leads to the accumulation of ROS-induced protein damages. The misfolded or aggregated proteins that cannot be successfully rescued by refolding, are delivered to the proteasome to be processed for proteolytic degradation. It is believed that age-related accumulation of oxidized proteins (Berlett et al., 1997) is due to increased protein damage, decreased removal and repair of damaged protein (Friguet et al., 2000; Petropoulos and Friguet, 2005). In eukaryotic cells, the degradation of oxidized protein is mainly achieved by the proteasome in both the cytosol and the nucleus (Grune et al., 2003).

The presence of very highly up-regulated genes on 'proteolysis and peptidolysis' in long-lived Hsp22+ flies strongly supports that Hsp22 could be involved in proteolytic activity resulting in lifespan alteration. Hsp22+ flies also display moderate but conserved up-regulation of genes involved in 'proteolysis and ubiquitin cycling', such as *CG7170* (*jon66Cii*), *CG32744* (*ubiquitin-5E*), *CG5266* (*pros25*), *CG1519* (*prosα7*) and *CG11981* (*prosß3*) (Table 5-5). The most important target site of degradation activated in Hsp22+ flies could be serine- or aspartic-type proteases. Because a number of genes are precisely involved in 'serine-type endopeptidase activity', and partly in 'NOT aspartic-type endopeptidase activity (*CG 13051*)'as shown in Table 5-2; 5-3; 5-5. Furthermore, in Hsp22 over-expressing flies, the most abundant protein was 'cathepsin D' with the ratio of 1.87 (65.1% of abundance in Hsp22 over-expressing flies versus 34.9% in common flies). This protein is one of aspartic-type (GAU, GAC) endopeptidases catalyzing hydrolysis of nonterminal peptide linkages in oligopeptides or polypeptides. This enzyme is involved in autophagic salivary gland cell death during *Drosophila* metamorphosis (Gorski et al., 2003), and flies mutated in this gene exhibit the key features of neuronal ceroid lipofuscinoses (NCLs) and an age-dependent neurodegeneration (Myllykangas et al., 2005). Besides, this enzyme has been associated with various biological processes such as apoptosis events (the release of

cytochrome c from mitochondria) (Roberg et al., 1999), the loss of the transmembrane potential, aging, Alzheimer's disease, and breast cancer.

During the aging process, the level and function of the proteasome/proteolysis system decrease and consequently damaged proteins tend to accumulate in the cells of aged organisms due to these degradation defects. Especially in the mitochondria, degradation of the oxidized protein adducts is mainly achieved by the ATP-stimulated Lon protease (Bota and Davies, 2002). Although it has been recently shown that mammalian mitochondria contain three major ATP-dependent proteases of Lon/ Clp-like and AAA proteases, the Lon protease is a better known, highly conserved protease found in prokaryotes and the mitochondrial compartment of eukaryotes (Bulteau et al., 2006). Additionally, the Lon protease is believed to play an important role in the degradation of oxidized mitochondrial matrix proteins such as aconitase, a TCA cycle enzyme (Bulteau et al., 2006; Bota and Davies, 2002; Bota et al., 2002). Down-regulation of the human Lon protease results in disruption of mitochondrial structure, loss in function, and cell death (Bota et al., 2005). Moreover, Lon-deficient cells reveal aberrant mitochondrial morphology and the presence of electron dense inclusion bodies in the mitochondrial matrix (Bulteau et al., 2006). All these observations imply the important role of the Lon proteolytic system for the degradation of the mitochondrial proteins and for the maintenance of mitochondrial structural and functional integrity.

Alterations in protein synthesis occur during embryonic development, cell growth, cell differentiation, and aging. Actually, a decrease in protein synthesis appears to be a universal phenomenon during normal aging process, and certain mutants for genes involved in ribosomal function have also a defective phenotype at developmental rate (Lawrence et al., 1986; Duffy et al., 1996; Saeboe-Larssen et al., 1998). It was also been reported that several cellular signaling mechanisms converge to influence protein synthesis process i.e. initiation of mRNA translation being a rate-limiting step and the most common target of translational control (Gingras et al., 1999; Proud, 2004). The IGF-1, TOR kinase, and p38 mitogen-activated protein kinase (MAPK) pathways play a key role in global control of protein synthesis (Proud, 2004; Wang et al., 1998). Rates of protein synthesis and activities of key mRNA translation factors decline with age in a variety of organisms (Rattan, 1996). In addition, results from *Drosophila melanogaster* show that poly-ribosome levels exhibit an

age-related decrease, although it is not clear whether such alterations are a consequence of adaptation to reduced mitochondrial function and energy production (Webster and Webster, 1983). Our results indicated that a number of genes related to the 'protein biosynthesis' are preferentially up-regulated in the Hsp22 over-expressing flies (Table 5-2; 5-3; 5-5). Especially, mitochondrial ribosomal subunit genes like *CG5904* (*mRpS31*), *CG6547* (*fs(1)Yb*), *CG5108* (*mRpS7*), and genes on 'translational initiation factor' and 'RNA cap binding / helicase activity' like *CG9075* (*eIF-4a*), *CG3186* (*eIF-5A*), *CG4035* (*eIF-4E*), *CG9177* (*eIF5*), *CG8280* (*Ef1α48D*) were partially involved in up-regulation.

Taken together, the increased expression of genes and proteins having functions in proteolysis and protein biosynthesis, indicates the possibility of additional factors influencing the extended lifespan in the Hsp22 over-expressing flies through protein degradation and protein turnover.

6.4 Hsp22 over-expressing flies have an enhanced oxidative phosphorylation

During the normal aging process, mitochondria appear to be particularly susceptible to damages especially by ROS. A number of studies carried out in several organisms have shown the morphological alterations of senescent mitochondria and a decline in the bioenergetic capacity with age, in an energy-demanding tissue specific way (Trounce et al., 1989; Yen et al., 1989; Wallace, 1992; Ferguson et al., 2005). Age-dependent decreases of mitochondrial functions have also been postulated by a dramatic decline in mitochondrial transcripts (16SrRNA), reduced activity of cytochrome c oxidase and ATP deficiency, but not mtDNA reduction (Calleja et al., 1993; Schwarze et al., 1998). Interestingly, a significant up-regulation of the genes related to the 'mitochondrial respiratory complex enzyme activity' or 'energy production' was observed in the comparative transcriptome of the long-lived Hsp22+ flies vs. the control flies.

In the process of mitochondrial oxidative phosphorylation, the electron-motive force through the mitochondrial inner membrane allows the protons to re-enter into the matrix, then to drive ATP synthesis (Walker, 1992). Complex I and complex III of the electron-transport chain are the major sites for ROS production (Sugioka et al., 1988; Turrens and Boveris, 1980). Moreover, interestingly, Cermelli et al. (2006) demonstrated that certain mitochondrial proteins (ATP synthase subunit beta, L-malate dehydrogenase, porin protein (DmVDAC)) were detected in the lipid-droplet fraction of an early *Drosophila* embryo. These proteins were over-expressed in the mitochondrial proteome of Hsp22 over-expressing flies, which may indicate a possibility of dynamic exchange of proteins between the lipid droplets and the mitochondria. Higher activity in parts of mitochondrial electron transport complexes in Hsp22 over-expressing flies is consistent with the results of increased expression of genes and proteins involved in mitochondrial energy metabolism. Thus, the Hsp22 over-expressing flies have remarkably higher activities of complex III regardless of ages than control flies, and the unit value of the complex III activity was the highest among the other complexes, indicating their major participating in ROS production as well as in ATP production.

6.5 Conclusion

In conclusion, the Hsp22+ flies have remarkably increased functions involved in complex mechanisms that decline during normal aging procedure such as mitochondrial energy generation, antioxidant activity, and protein turnover. On the basis of overall observations (as summarized in Table 6-3) in this study, potential mechanisms of longevity in the flies over-expressing Hsp22 are suggested in Fig. 6-1. The Hsp22 over-expressing flies have a higher energy production than control flies. However, larger energy production can lead to the increased ROS generation and increased damages to proteins or molecules in the cell. These damages can be protected by an enhanced antioxidant activity, or can be regulated by protein folding (chaperone-like) activity or by proteolysis activity to remove damaged protein. These chaperone or proteolysis processes may need greater energies that can be supplied in the Hsp22 over-expressing flies having larger oxidative phosphorylation activity. Consequently, the activated protein synthesis process will rapidly replace the damaged proteins using the amino acids released by protein degradation.

Especially, if these mechanisms are applied to control a quality of mitochondrial proteins with a chaperone-like activity of Hsp22 in the mitochondrial matrix, and thus enable to properly maintain the mitochondrial metabolic functions, they may preferentially increase cellular homeostasis and longevity. Possibly, the up-regulation of *hsp22* and co-transcriptional regulation of the associated genes, as well as their corresponding proteins and their post-translational regulation involved in these mechanisms may be associated, and play a role as positive regulators of lifespan. Further assessment of the postulated associations with genes/proteins and Hsp22-dependent activities or regulation on proteolysis, antioxidation, and protein biosynthesis will be able to confirm the key roles of chaperone-like Hsp22 to maintain overall homeostasis in cellular system.

Table 6-3. Conserved functions specific in Hsp22+ flies among the proteomic, transcriptomic and physiological analysis

Proteome	Transcriptome	Physiological activity
Mitochondrial energy metabolism ↑ (oxidative phosphorylation, TCA cycle)	Mitochondrial energy metabolism ↑ (oxidative phosphorylation, TCA cycle)	Respiratory complex enzyme activity ↑
Antioxidant,detoxification enzyme ↑	Antioxidant genes ↑	Stress resistance ↑ (oxidative stress & starvation), protein oxidation ↓ during aging
Proteolysis ↑	Proteolysis ↑	
Fatty acid oxidation ↑	Lipid/hormonal metabolism ↑	
	Protein biosynthesis ↑ (ribosomal constituent)	

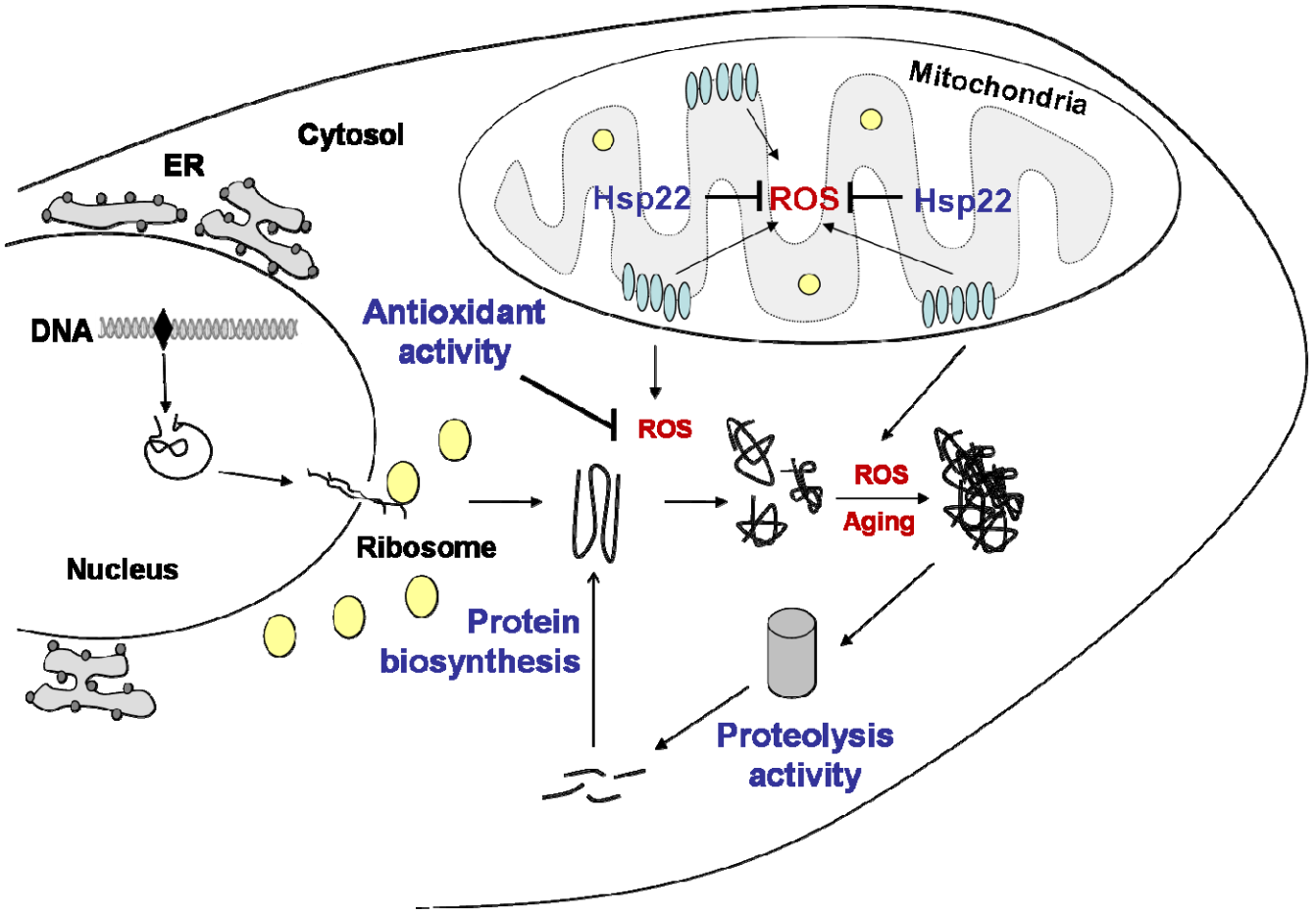


Fig. 6-1. Potential mechanisms of longevity in flies over-expressing Hsp22

Chaperone-like activity of Hsp22 in the mitochondrial matrix could contribute to the quality control of cellular proteins directly or indirectly by increasing protein turnover/antioxidant activity. This would in turn enable to properly maintain the mitochondrial metabolic functions and cellular homeostasis which are crucial for longevity.

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