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The activation of the heat shock response by Cyclin G/ cdk5 complex after cold stress and the *Wolbachia* influences on cellular stress response in *Drosophila melanogaster*

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1. Riassunto

I cambiamenti climatici causati dall'attività umana continuano a minacciare la salute della biosfera, con pesante impatto sugli ecosistemi, colpendo tutti i livelli trofici, dalle piante agli insetti, destabilizzando le dinamiche di popolazioni che possono portare all' estinzione della specie. La temperatura ha sempre influenzato la distribuzione e l'abbondanza delle specie. Per gli ectotermi, essa ne influenza la distribuzione, lo sviluppo, la crescita oltre che i loro processi fisiologici e metabolici. Evidenze crescenti hanno dimostrato che stress fisici, fisiologici e patologici scatenano la risposta cellulare allo stress in tutti gli organismi. Essa comprende l'autofagia, la risposta allo stress del reticolo endoplasmatico e del mitocondrio, la risposta da danno al DNA e la risposta heat shock. Tutti questi meccanismi sono regolati sia indipendentemente sia in modo coordinato. La risposta cellulare allo stress è considerata un "tutore" della omeostasi cellulare. Molte sono le proteine "ponte" in essa coinvolte, di cui molte sono già state identificate ma ne restano ancora da indentificare.

Attualmente la risposta allo stress dell' organismo ha attirato enorme attenzione dato che, in questa Era, diverse e a volte severe condizioni di stress mettono a dura prova l'omeostasi cellulare.

Questo è il contesto in cui nasce questo progetto di ricerca volto a scoprire nuovi geni coinvolti nella risposta allo stress, a comprendere come gli organismi reagiscano ai cambiamenti di diversi fattori ambientali come per esempio al freddo. Inoltre in questo lavoro si cerca di capire se e quale ruolo abbiano le infezioni batteriche sulla risposta allo stress da freddo. Per tutti questi scopi è stato scelto come modello sperimentale la *Drosophila melanogaster* . All'inizio è stato scelto di studiare alcuni geni chiave di alcuni meccanismi cellulari coinvolti nella risposta cellulare allo stress, dopo stress da freddo. Essi sono: Atg1 per l'autofagia, Xbp1 per la risposta allo stress del reticolo endoplasmatico e Hsp70Aa per la risposta heat stress. Dai risultati ottenuti si è visto che tutti i geni sono influenzati dallo stress da freddo. Successivamente è stato deciso di concentrare

l’attenzione sulla risposta heat stress e la sua produzione delle proteine Heat shock. Tra le proteine Heat shock, la famiglia Hsp70 ha un ruolo chiave nella coordinazione della risposta cellulare allo stress. In particolare sono state ricercate proteine “ponte” tra i complessi ciclina/cdk, che regolano sia la progressione del ciclo cellulare che la trascrizione di alcuni geni, e la risposta cellulare allo stress. Si è scelto di fare uno studio dell’espressione temporale del fattore di trascrizione delle proteine heat shock, HSF, della Ciclina G, della cdk5 in aggiunta a Hsp70Aa, dopo diverse ore di stress e di recupero. Hsp70Aa, la ciclina G e la cdk5 risultano molto influenzati dallo stress da freddo. Invece i livelli di HSF restano pressoché costanti. Successivamente, per verificare l’interazione della ciclina G con la cdk5 sono stati condotti esperimenti di co-immunoprecipitazione. E’ stata osservata l’interazione tra le due proteine da trenta minuti a due ore di recupero dopo lo stress da freddo. Dopo questo risultato si è deciso di studiare il coinvolgimento della cdk5 nella regolazione

trascrizionale di Hsp70Aa, una isoforma della famiglia delle Hsp70. A questo scopo la cdk5 è stata silenziata mediante l'esperimento di RNA interference, condotto usando il sistema GAL4/UAS. E' stato osservato che la cdk5 regola la trascrizione di Hsp70Aa. Questo risultato è stato confermato stressando le *D. melanogaster* con il gene cdk5 silenziato. Queste hanno esibito una risposta attenuata allo stress da freddo mostrando livelli ridotti di Hsp70Aa rispetto alle linee di controllo, stressate allo stesso modo. Inoltre è stata studiata la possibile influenza dell'infezione da *Wolbachia* (il piu' diffuso endosimbionte trovato negli artropodi) sulla risposta cellulare allo stress oltre all'influenza, che è già nota in letteratura, sul metabolismo, il sistema immunitario e la riproduzione. A questo scopo sono state considerate due linee di *D. melanogaster* affette o meno dall'infezione da *Wolbachia* ed è stato studiato se i meccanismi di autofagia, di risposta allo stress del reticolo endoplasmatico e la risposta heat stress fossero influenzati dall'infezione da *Wolbachia*. Lo studio è

stato condotto studiando i livelli di mRNA per i geni chiave dei tre meccanismi cellulari presi in esame, già precedentemente descritti. Tutti risultano essere influenzati da *Wolbachia*. Inoltre essa influenza la fase di recupero dallo stress da freddo. Per finire, è stato studiato l'effetto dello stress da freddo sull'espressione di un gene codificante una proteina di superficie di *Wolbachia*: il gene wsp. Facendo una RT-PCR è stato visto come lo stress da freddo abbia un effetto negativo sull'espressione di questo gene fino a 8 ore di recupero dallo stress da freddo indicando che *Wolbachia* è molto sensibile a questo stress anche quando l'organismo ospite è in fase di recupero.

2. Summary

The environmental changes caused by the human activities continue to threaten the health of the biosphere with heavy impact on natural ecosystems affecting all trophic levels from plant to insect and destabilizing the population dynamics that can lead to the extinction of the species. The temperature has influenced the distribution and the abundance of all species. As regards ectotherms, the temperature affects their distribution, development and growth in addition to physiological and metabolic processes. Increasing evidences have shown that, in all organisms, environmental, physiological and pathological stresses evoke the cellular stress response (CSR) involving as example the autophagy, the unfolded protein response, the DNA damage response, the mitochondrial unfolded protein response and the heat shock response to maintain and restore or re-establish the cellular homeostasis. Evidences showed that these pathways are regulated independently as well as co-ordinately as network of interlinked pathways.

The CSR is considered the "guardian" of cellular homeostasis, it is a complex mechanism that involves many cellular pathways and cross-talker proteins. Afterward, the identification of the linker proteins that coordinate and regulate these interactions is very important. Nowadays many genes implicated in the CSR have been identified, but a lot of gaps need to be filled clarifying the determined

functions and the interaction between genes of CSR pathways.

In this Era, the CSR captures big attention because, the organisms are continually threatened by different stresses conditions.

Within this context, this research project was thought in order to find new candidates genes involved in these cross-talk pathways, with the aim to understand how the organisms react to environmental factors e.g. cold stress and to deepen the influence of infection on the cellular stress response. We choose *D. melanogaster* as experimental model organism to study some keys genes involved in cellular stress response e.g. the Atg1 gene for the autophagy, the Xbp1 for the endoplasmic reticulum unfolded protein response, and the Hsp70Aa for the heat shock response after the cold stress. All the genes studied, were found to be involved in the stress response after cold stress. Hence, it was decided to focus the attention on the heat shock response and in particular on the heat shock proteins of the Hsp70 family since have a key role in the coordination of the CSR. The research continued searching a link between the cell cycle checkpoints, where cyclin/cdk complexes are involved, and the cellular stress response. With this aim it was done a time-course analysis of the expression of Hsp70Aa, HSF (the transcriptional factor of the heat shock proteins), Cyclin G and cdk5 upon different

protocols of stress induction and recovery. All the analysed genes changed in gene expression more during the recovery phases than the stressing period except for HSF. Next step was to verify the interaction between the cyclin G and the cdk5. For this purpose co-immunoprecipitation experiments were set up. The interaction between these two proteins was confirmed. Then it was studied the possible involvement of the cdk5 in the activation of heat shock response after cold stress. Through RNAi experiment, using the GAL4/UAS system, it was demonstrated that cdk5 regulates the Hsp70Aa transcriptional activation.

Moreover it was investigated on the possible involvement of *Wolbachia* infection (the most widespread endosymbiotic microbe found in arthropods) on the cellular stress response.

It is already known that the infection by this bacterium affects the metabolism, the immunity system and reproduction. The gene expression of Atg1, Xbp1 and Hsp70Aa was studied on *D. melanogaster* strains infected or not by *Wolbachia* upon cold stress. Results showed that *Wolbachia* infection affects the expression levels of these genes and the recovery phase after cold stress treatments. At last it was studied the effect of the cold stress on the *Wolbachia* infection by monitoring the expression of the gene encoding for a *Wolbachia* surface protein (wsp). It was seen that the cold stress affects negatively the wsp gene expression

up to 8 hours of recovery after cold stress, indicating that *Wolbachia* is very sensitive to cold stress and that its activity is hampered even after recovery of the host.

3. Introduction

3.1.1. The cellular stress response

Always the cells of all organisms are exposed to different array of physiological, environmental pathological and physical stresses. Hence, organisms evolved the cellular stress response to preserve cellular homeostasis. The cellular stress response (CSR) is a universal network of pathways that identify, check and respond to stresses. Through the evolution, all organisms had conserved the proteins involved in key aspects of the CSR (Morimoto and Santoro, 1998; Jolly and Morimoto, 2000; Kültz, 2005). The CSR includes the unfolded protein response (Stevens and Argon, 1999), the autophagy (Cuervo, 2004), the heat shock response (reviewed in Morimoto, 2008), the mitochondrial unfolded response, and nuclear DNA damage response (Kourtis and Tavernarakis, 2011) (Fig.3.1.).

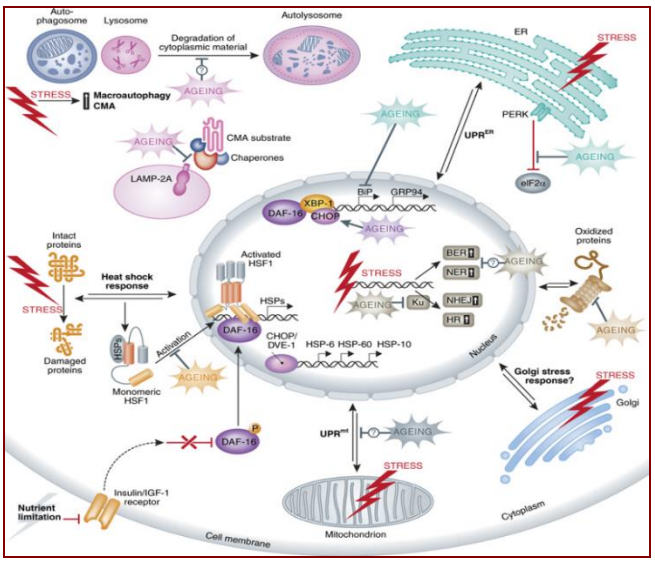

Fig. 3.1. General and organelle-specific stress response pathways. Depending on the type of macromolecule and the site of damage, distinct stress response pathways, such as autophagy, heat shock response, UPR^{mt}, UPR^{ER}, remodelled proteasome and the DNA damage response are initiated. Double arrows denote bi-directional communication with the nucleus, which

involves generation of stress signals in the stressed organelle or the cytoplasm, transduction of the signals to the nucleus and up-regulation of stress-relieving proteins, which in turn function to ameliorate damage. Although, a typical Golgi stress response pathway has not been described yet, several types of stress may influence gene expression in the nucleus and cell homeostasis by impinging on Golgi function. BER, base-excision repair; BiP, Ig-binding protein; CHOP, C/EBP homologous protein; CMA, chaperone-mediated autophagy; DAF-16, abnormal dauer formation 16; DVE-1, defective proventriculus 1; GRP94, glucose-regulated protein 94; HSF1, heat shock factor 1; HSP, heat shock protein; HR, homologous recombination; IGF-1, insulin growth factor 1; LAMP-2A, lysosome-associated membrane protein 2A; NER, nucleotide-excision repair; NHEJ, non-homologous end joining; PERK, PKR-like ER kinase; $UPR^{ER/mt}$, unfolded protein response endoplasmic reticulum/mitochondrion; XBP-1, X-box-binding protein 1 (Kourtis and Tavernarakis, 2011).

3.1.2. The unfolded protein response

In the eukaryotic cell, the endoplasmic reticulum (ER) provides to new synthesis, folding of secretory and membrane proteins and to their post-transnationally modification before exit from the organelle. The ER activities change in response to physiologically cellular request or in response to cell differentiation or environmental changing (Lai et al., 2009; Ron and Walter, 2007). Moreover, these cellular requests can increases the misfolded proteins in the ER compartment that adding to other perturbation that can occur (as alteration in redox state or in calcium levels) evoking ER stress. Stressing conditions can be produced by some pharmacologically treatments also. When the UPR^{ER} is activated, the cells temporarily inhibit the new proteins synthesis promoting the transcription of the proteins involved in the unfolded proteins response (UPR^{ER}) (Fig. 3.2.).

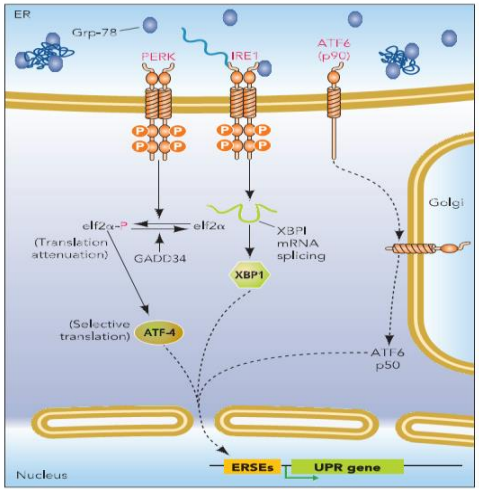

Fig. 3.2. UPR signaling pathways in mammalian cells. The UPR is mediated by three ER-resident transmembrane proteins that sense ER stress and signal downstream pathways. The PERK kinase is activated by dimerization and phosphorylation. Once activated, it phosphorylates eIF2, resulting in translation attenuation. Phosphorylated eIF2 selectively enhances translation of the ATF4 transcription factor that induces expression of UPR target genes. Activation of IRE1 by dimerization and phosphorylation causes IRE1-mediated splicing of XBP1 mRNA. Translation of spliced XBP1 mRNA produces a transcription factor that upregulates target genes via the ERSE promoter. ATF6 activation involves regulated intramembrane proteolysis. The protein translocates from the ER to the Golgi where it is proteolytically processed to release a 50-kDa transcription factor that translocates to the nucleus and binds the ERSEs of UPR target genes. All three ER-resident transmembrane proteins are thought to sense ER stress through Grp78 binding/release via their respective lumenal domains, although structural studies have also suggested that IRE1 may interact with unfolded proteins directly. The GADD34 protein, a protein phosphatase upregulated by the PERK pathway, dephosphorylates eIF2α to restore global protein synthesis (Lai et al., 2007).

The X box binding protein-1 (Xbp1), the Activating Transcriptional Factor 4 (ATF4) and the Activating Transcription Factor 6 (ATF6) are the transcriptional factor regulating the transcription of the UPR genes. These are involved in the preservation of the ER folding capacity, in the prevention of the formation and the aggregation of misfolded proteins, and in the promoting the breakdown of misfolded proteins (Kourtis and Tavernarakis, 2011; Lai et al., 2007; Ron and Walter, 2007; Rasheva and Dominigos, 2009). If the homeostasis is not be restored, the apoptosis occurs (Lai et al., 2007; Ron and Walter, 2007).

3.1.3. The autophagy

Autophagy (from the Greek for *self-eating*) is a ubiquitous cellular pathway in eukaryotic cells that work through the formation of double membranes vesicles called autophagosome, sequestering organelles, proteins and cytoplasm portion and in fusion with lysosome creates autolysosome where the sequestered component are degraded by hydrolase (Yorimutsu et al, 2006) (Fig. 3.3.).

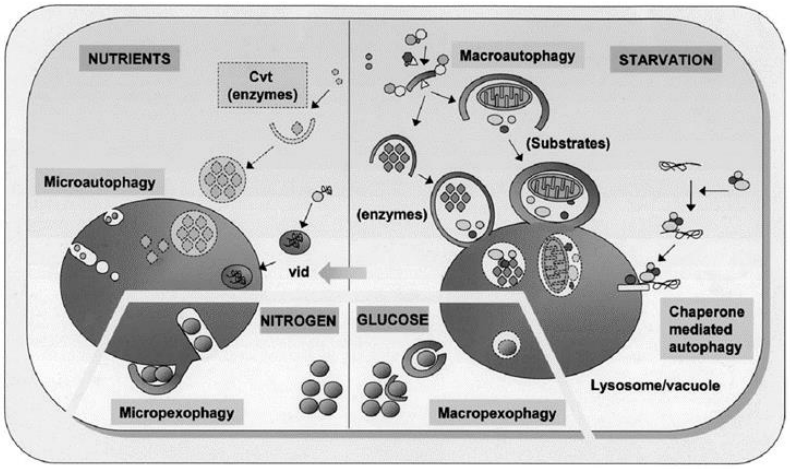

Fig. 3.3. Pathways for the degradation of intracellular proteins in lysosomes. A schematic model of the different mechanisms that lead to degradation of intracellular proteins in lysosomes and the stimuli that maximally activate them are depicted. The arrow indicates activation of vid after switching from poor to rich nutritional conditions. The cytosol to vacuole (cvt) pathway that transports hydrolases is also shown (dotted) because during the nutrient deprivation transport of the same enzymes is performed by macroautophagy (Cuervo, 2004).

Physiologically this mechanism is implicated in the normal turnover and recycling of long live cellular components and damaged proteins, organelles. Many aspects of homeostasis and development are regulated by autophagy (Levine, 2005; Lum et al., 2005; Rubinsztein et al., 2007; Shintani and

Klionsky, 2004). In the same way, cells activates this pathway under food deprivation conditions organism (starvation) and for the nutrients acquisition. Autophagy also was essential for the animal development. In fact during *Drosophila melanogaster* metamorphosis, autophagy operates to the large-scale histolysis that breaks down larval tissues and makes for the formation of adult structures (Butterworth et al., 1988; Lee and Baehrecke, 2001; Lee et al., 2002). Likewise this mechanism is important for the development and morphogenesis of vertebrates (Cecconi and Levine, 2008; Melendez and Neufeld, 2008). Recently it was given importance to the implication of the autophagy in themselves cellular protection despite environmental stresses. Multiple reports show that the autophagy-related genes (Atg) expression can be stimulated in response to different array of cellular stresses such as DNA damages and intracellular pathogens (Girardot et al., 2004; Thorpe et al., 2004; Xiong et al., 2007, Wu et al., 2009, Kourtis and Tavernarakis, 2011; Yorimutsu et al, 2006, Kromer et al. 2010). Autophagy plays alone as in cooperation with other cellular stress response pathways (Wu et al. 2009). In *Saccharomyces cerevisiae* almost 20 Atg genes were discovered many of which are preserved in metazoan (Scott et al. 2007). The Atg1 gene is a serine threonine enzyme evolutionary conserved (Jung et al, 2010). *Drosophila melanogaster* has the Atg1 homologue

also. Atg1 interacts with many component of the autophagy pathway so has a central role in the coordination of the autophagy response (Scott et al. 2007), (Fig. 3.4.).

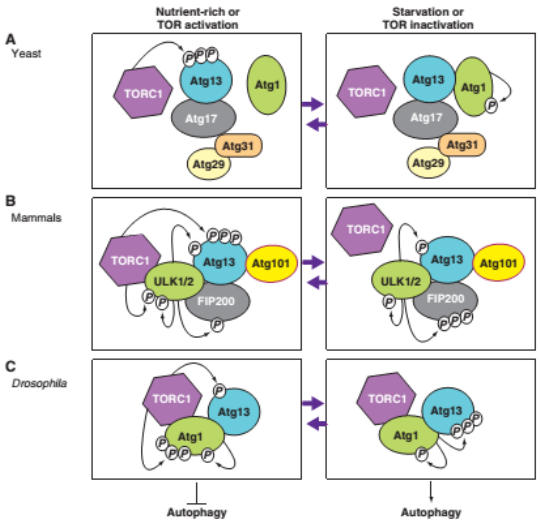

Fig. 3.4. Dynamics of Atg1 complexes upon autophagy induction in different eukaryotes. (A) In yeast, under nutrient-rich conditions, the active TOR complex 1(TORC1) hyperphosphorylates Atg13 (Kamada et al., 2010). This prevents the association of Atg1 with Atg13, which is bound to Atg17, Atg31 and Atg29, leading to inhibition of autophagy induction. Under starvation conditions when TORC1 is inactivated, Atg13 is no longer phosphorylated by TORC1, whereas Atg1 is autophosphorylated, leading to the association of Atg1 with the complex between Atg13, Atg17, Atg31 and Atg29, and subsequent autophagy induction (Cebollero and Reggiori, 2009; Chang and Neufeld, 2010; Kamada et al., 2010; Nakatogawa et al., 2009). (B) In contrast to yeast, mammalian ULK (ULK1 or ULK2, the homologs of yeast Atg1) forms a stable complex with mammalian Atg13, FIP200 (a putative counterpart of yeast Atg17) and Atg101 (an Atg13-binding protein), irrespective of TORC1 activation. Under nutrient-rich conditions, the active TORC1 associates with the ULK complex (ULK1 (or ULK2)–Atg13–FIP200-Atg101), phosphorylates ULK1 (or ULK2) and hyperphosphorylates Atg13, which inhibits the kinase activity of ULK1 (or ULK2) and thus blocks autophagy induction. Under starvation conditions when TORC1 is inactivated, TORC1 dissociates from the ULK complex, preventing phosphorylation of Atg13 and ULK1 (or ULK2) by TORC1 and leading to autophagy induction, whereas ULK1 (or ULK2) still phosphorylates Atg13 and itself, and hyperphosphorylates FIP200 (Chang and Neufeld, 2010; Mizushima, 2010; Yang and Klionsky, 2010). (C) Similar to the situation in mammals, in Drosophila Atg1 forms a complex with Atg13 irrespective of TORC1 activation (Chang and Neufeld, 2010). Under nutrient-rich conditions, the active TORC1 phosphorylates Atg13 and hyperphosphorylates Atg1, leading to the inhibition of autophagy induction. Under starvation conditions, when TORC1 is inactivated, Atg1 and Atg13 are no longer phosphorylated by TORC1, whereas Atg1 still phosphorylates itself and hyperphosphorylates Atg13, leading to autophagy induction. Figure modified from Chang and Neufeld (Chang and Neufeld, 2010) with permission (Yongqiang and Klionsky, 2011).

However, how the autophagy machinery senses and responds to stress is not thoroughly understood. Such regulation could occur at several levels, as autophagy can be regulated by

transcriptional, as well as post-transcriptional mechanisms. Consistent with a function of gene regulation in this context. A misregulation of this process is associated with a great numerous pathologies i.e. cancer (Høyer-Hansen & Marja Jäättelä, 2008; Mathew et al., 2007; Yue et al., 2003), neurodegeneration (Hara et al., 2006; Komatsu et al., 2006), muscular atrophy (Mammucari et al., 2007; Zhao et al., 2007). Moreover this mechanism is involved in cell death, pathogenic infection, neurodegenerative disease, cell growth and stress response and represent the most protective cellular mechanism through cell fight against degenerative and neoplastic disease and infections (Scott et al., 2007; Levine and Kroemer, 2008; Mizushima et al., 2008).

3.1.4. The mitochondrial unfolded protein response

Mitochondria are cellular organelles essential for numerous metabolic processes as ATP production, Calcium signaling, iron-sulfur cluster biogenesis, apoptosis, nucleotide and amino acid metabolism. Mitochondrial proteins are encoded by nuclear genes in addiction to mitochondrial genes. Proteins are imported into mitochondria by translocases in the inner and/or outer membranes with the

help of mitochondrial chaperones belonging to Hsp70 and Hsp60 families (Neupert 1997; Broadley and Hartl, 2008). The electron transport chain activity produces Reactive Oxygen Species (ROS) as changing in environmental temperatures and exposure to toxin increase the accumulation of misfolded or unfolded mitochondrial proteins with potentially deleterious effects on the mitochondrial genome's susceptibility that can acquires mutations. Thus cells elicit the mitochondrial unfolded protein response (UPR^{mt}) as results to the accumulation of unfolded or misfolded proteins beyond the organelle's chaperone capacity (Pellegrino et al., 2013), inducing nuclear gene transcription of mitochondrial chaperones that preserve proteins mitochondrial homeostasis (Pellegrino et al., 2013; Haynes et al., 2010; Bukau et al., 2006; Young et al., 2004; Tatsuta and Langer, 2008; Baker et al., 2011). In mammals cells c-Jun is the transcriptional factor activated by c-Jun-N terminal kinase (JKN) that promote the transcription of mitochondrial chaperones (Horibe and Hoogenraad, 2007). Mammalian JNKs, such as p38 MAPKs, was activated by different array of stresses (Pelech, 1996; Goberdhan and Wilson, 1998), likewise ultraviolet (UV) and X-irradiation (Dérijard et al., 1994), heat shock (Adler et al., 1995), oxidative and chemical stresses (Cavigelli et al., 1996; Liu et al., 1996) (Fig. 3.5.).

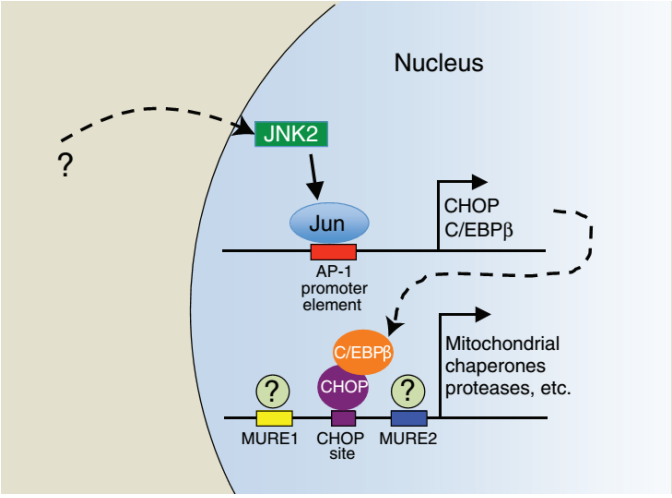

Fig. 3.5. Mitochondrial UPR signaling in mammalian cells. Transcriptional induction of mitochondrial chaperone and protease genes in mammalian cells requires several transcription factors, with CHOP and C/EBP b having important roles. CHOP binding sites along with two other conserved regions (MURE1 and MURE2) are found in a number of genes that are induced in response to mitochondrial stress (Aldridge et al., 2007). The identity of the transcription factors that interact with MURE1 and MURE2 are currently unknown. CHOP is itself transcriptionally induced in response to mitochondrial stress, which represents an early event in the pathway (Horibe and Hoogenraad, 2007). CHOP induction requires the kinase JNK2 and the transcription factor Jun, which binds to the AP-1 site within the promoters of the CHOP and C/EBP b genes. Signaling inputs that indicate mitochondrial stress and lead to activation of JNK2 signaling are currently unknown, but the up-regulation of mitochondrial chaperone and proteases re-establishes protein folding homeostasis within the organelle (Haynes and Ron, 2010).

D. melanogaster has the same conserved pathway in which the mammals c-Jun transcription factor and JNK kinase homologues are respectively d-Jun and d-JNK (Goberdhan and Wilson, 1998) and *D. melanogaster* dJNK pathway cascade is involved in stress response also (Botella et al., 2001). More disease are linked with mitochondrial dysfunction e.g. Parkinson disease, Friedreich's ataxia and cancer. Moreover mutation in the mitochondrial genome causes many disorders such as Leigh Syndrome and Leber's hereditary optic neuropathy (Wallance, 2005).

3.1.5. The heat shock response

A key aspect of CSR is the heat shock response e.g. the production of heat shock proteins (review, Morimoto et al., 2000). In 1962 Ritossa observed that high temperature induce puffs (regions of high transcription activity) in polytene chromosome of *Drosophila melanogaster* larvae salivary glands. After it was seen that the heat shock proteins were interested to an increased transcription activation (Lindquist 1986). Next studies shown that the heat shock response is a pathway conserved from bacteria to animals and plants that preserve cellular homeostasis by oxidative stress, heat shock, heavy metals producing the heat shock proteins (Hsps) (Craig, 1985; Lindquist and Craig, 1988). The Hsps are classified on the basis of molecular mass and their sequence homology and function in Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small Hsps. The Hsps, also known as molecular chaperones, are involved in folding, translocation of the proteins in different cellular compartments and demolition of proteins aggregate (Nover and Scharf 1984; Feder and Hofmann, 1999; Sørensen et al., 2003; Parsell et al., 1993).There are some Hsp constitutively present in cells (heat shock cognate) while other Hsp are expressed only after a plethora of stress signals such as heat shock, heavy metals, oxidative stress, inflammation (Hartl and Hayer-Hartl 2002). The stressing agents affect the hydration and the redox state

of the cell causing the increase of misfolded proteins in cellular compartment and altering biological processes (Jolly and Morimoto 2000). Moreover the Hsp are involved in physiological and non-stressed processes as cell cycle, cell proliferation and differentiation (Milarski and Morimoto, 1986; Jerome et al., 1993; Hang et al., 1995), (Fig.3.6.).

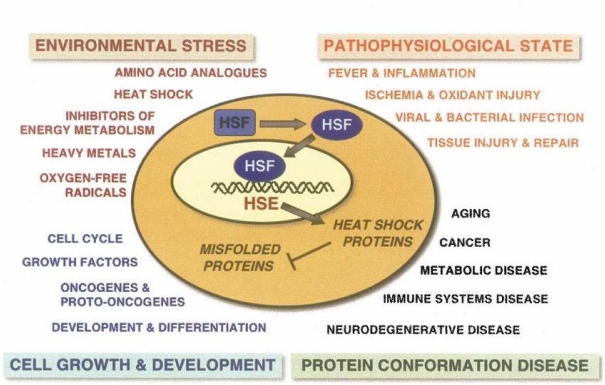

Fig. 3.6. Cell stress response. The expression of HS genes including chaperones and components of the clearance machinery is induced in response to three classes of physiological and environmental stress conditions including environmental stress, pathophysiological stress, and protein conformational disease, and by a fourth class of cell growth and development. Indicated *below* each major class are representative conditions known to involve the expression of heat-shock proteins and chaperones (Morimoto, 2008).

In arthropods, many Hsps are up-regulated in response to heat and cold stress in addition to heavy metals, ethanol and desiccation (Hoffmann et al., 2003; Hoffmann et Parsons 1991; Tammariello et al 1999). The Hsp70 family includes the most widely studied Hsps under cold stress conditions; in particular, the isoform Hsp70Aa has been shown to play an important role in the response to thermal stress in *D. melanogaster* (Colinet et al., 2009).

Heat stress or other stress induce the Hsps gene transcription by a group of transcriptional factors named Heat shock factors (Hsf) that bind the heat shock elements (HSEs) and promote the Hsp transcription. The HSE consists of a pentameric sequence nGAAn inverted repeat at last three times nTTCnnGAAnnTTCn upstream all the Hsps genes (Wu et al., 1994). Among these Hsfs, in vertebrate and plants were identified four Hsf: Hsf1, Hsf2-3-4. The role of each Hsf resulted incomplete. Despite vertebrate, the invertebrates have only one Hsf (Akerfelt et al., 2010).The vertebrate Hsf1 is functional analogue to the *D.melanogaster* and yeast Hsf (Nakai and Morimoto, 1993; Rabindran et al., 1991; Sarge et al., 1991). The molecular mechanism of Hsp transcriptional activation is a multistep process in which the Hsfs need to be phosphorylated, translocated from cytosol to nucleus, where the Hsf trimer formation has been done, and through its binding to heat shock element (HSE) starting the Hsp genes transcriptions. The enzyme involved in the Hsf1 phosphorilation is the protein kinase C (PKC) that phosphorylate proteins on serine threonine residues. The proposed mechanism of Hsp70 auto-regulation was showed in fig 3.7. Different types of stress causes an increases of Ca^{2+} endocellular level that bind and activate the PKC, and the phosphatise (PP), inhibiting. After the PKC phosphorylate Hsf1 activating that came in the nucleus, and

after trimerization binds the HSE element of Hsps genes activating their transcription.

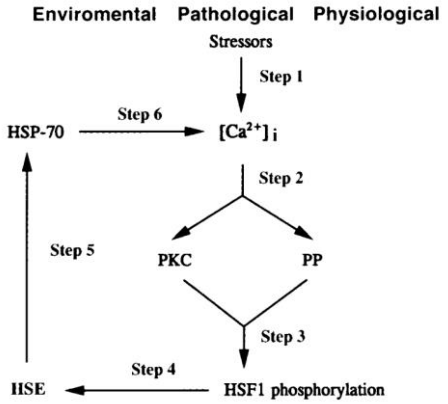

Fig. 3.7. Proposed auto-regulation of HSP70 through PKC and PP. Stressors cause an immediate increase in cytosolic free Ca^{2+} concentration $[Ca^{2+}]i$ (step 1), which activates PKC activity and inhibits PP activity (step 2). This leads to HSF1 phosphorylation (step 3) and binding to HSE (step 4), which induces an increase in HSP-70 production (step 5). This overexpression of HSP-70 is capable of inhibiting the increase in [Ca2]i (step 6) and subsequent steps (Ding et al., 1998).

It was also demonstrated that the overexpression of Hsp70 has a negative regulation on Hsf1 activation (Ding et al., 1998). For this reason it was not observed the Hsp70 overexpression after other stimulations. Despite PKC is the serine threonine enzyme involved in the Hsp transcription activation, the increased evidence shown that environmental stresses activate many pathways, so is not exclude that other enzyme are involved in Hsp regulation (de Pomerai et al., 2008).

The Hsp were overexpressed in more pathologies as Alzheimer's disease, epilepsy, Parkinson disease, amyotrophic lateral sclerosis, in tumors and pathologies

related to autoimmune disease as multiple sclerosis (Turturici et al., 2011). In *D. melanogaster* the Hsp70 overexpression causes the decrease growth, development and survival to adulthood (Krebs and Feder, 1997). Hence, it is important to understand this cellular response pathway for identification of prevention methods and for the treatment of these disease state.

3.1.6. The nuclear DNA damage response

It is known that the stress kinase pathways is activated after cellular and cytoplasm component damage while the nuclear damage response is activated after a DNA damage (Amanda et al., 2009). DNA damage can be evoked by different stresses e.g. UV irradiation, reactive oxygen species (ROS) (Lindahl, 1993; De Bont and van Larebeke, 2004; Hoeijmakers, 2009). The genome stability was preserved by a machinery of repair that respond to various DNA damage (Fig. 3.8).

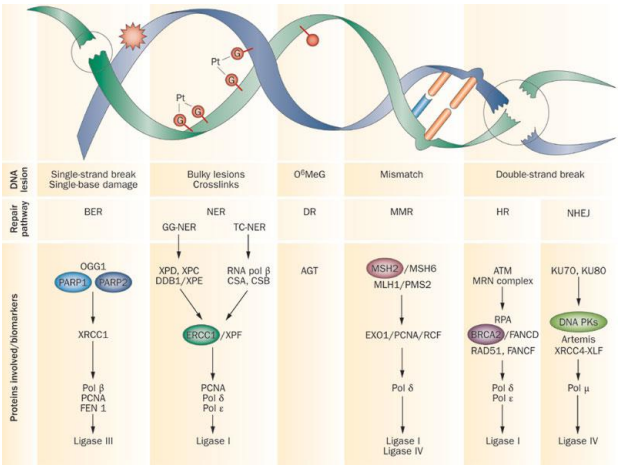

Fig. 3.8. Main DNA lesions and corresponding DNA-damage-repair pathways. DNA lesions that affect a single strand without significantly disrupting the helical structure are generally repaired by BER, whereas DNA damage significantlydistorting the DNA helix is repaired by NER. DR copes with small chemical changes affecting a single base, and MMR repairsmismatches in the pairing of DNA caused by replication errors. Finally, HR and NHEJ, although distinct pathways, are both involved in the repair of DNA double-strand breaks: HR allows 'error free' repair of the lesion whereas NHEJ is an 'errorprone' mechanism that repairs DNA but at the cost of introducing mutations into the genome. 8 The selection of HR or NHEJ is primarily based on the phase of the cell cycle and the expression, availability and activation of DNA-repair proteins. 104 Abbreviations: AGT, O6-alkylguanine-DNA alkyltransferase; ATM, ataxia telangiectasia mutated; BER, base excision repair; DR, direct repair; GG-NER, global genome NER; HR, homologous recombination; 06MeG, O6-methylguanine; MMR, mismatch repair; NER, nucleotide excision repair; NHEJ, non-homologous end joining; TC-NER, transcription-coupled NER (Vinay et al., 2012).

Studies on DNA damage focused on the cell response under damage induced by ionizing radiation or highly reactive chemicals. Recently it was demonstrated that osmotic stress and heat shock also causes DNA damage and the DNA repair mechanisms were activated (Kültz and Chakravarty, 2001; Kültz et al., 1998; Seno and Dynlacht, 2004).The types of DNA damage can be grouped in: DNA single-strand breaks (ssb), DNA double-strand breaks (dsb), base modification and DNA nucleotide adduct formation, mismatches of DNA bases. The mechanisms to repair the damage to single base or nucleotide are respectively base excision repair (BER), nucleotide excision repair (NER). The Base excision repair

(BER) is a pathway occurring for the repair of a damaged single base on a single DNA strand while the other strand acts as the template for the repair (Krokan et al., 2000). The nucleotide exition repair (NER) consists in the remotion of DNA lesion caused by UV irradiation or chemicals agents (Sancar et al., 2004) while, the ionizing radiations and chemical substance produce reactive oxygen species (ROS) forming double-strand breaks that are repaired through homologous recombination (HR) or nonhomologous end-joining (NHEJ) and nucleotide mismatch repair (MMR).These DNA repair mechanisms are more conserved between prokaryotic and eukaryotic organism (for details see Sancar et al., 2004). Many pathologies are related to DNA damage repair system failure, as example the Xeroderma Pigmentosum, the Cockayne syndrome, are related to NER failure, moreover the association between inflammation and oxidative stress is well documented (Wiseman and Halliwell, 1996; Halliwell and Gutteridge, 1996). Recently it was find a link between the defects in repair of oxidative DNA damage and a predisposition to disease. However has not been easy to link the DNA damage and mutation to the pathophysiological changes (Cooke et al., 2003).

3.2. The Cyclin and the kinase cyclin dependent complex

Recent evidences underline many links between the stress-response and checkpoint pathways in normal cell growth, development as in the response to UV ray, oxidative stresses. Moreover both pathway can regulate the cellular stress response also regulating the transcription factor that control the genes involved in stress response (Amanda et al., 2009).

Cyclins are a family of proteins containing a domain of 100 amino acid called “cyclin box” extremely conserved in all organism (Pines and Hunter, 1989; Nugent et al., 1991). Through the cyclin box some cyclins act as regulatory subunit of cyclin dependent–kinase (cdks) forming an active kinase complex that phosphorylates cellular substrate on serine and threonine residues influencing biological processes (Hunt, 1991; Pines, 1991). Just the cyclins A, B, D and E are involved in the cell cycle progression (Fig. 3.9.) while the cyclins C, K, H and T are involved in transcription regulation. The Cyclins I, S and F are involved in proteolysis, cell survival and short term memory (Faradji et al., 2011).

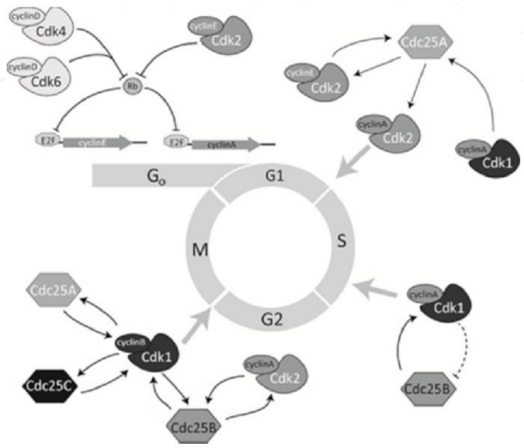

Fig. 3.9. Positive feedback loops between Cdk-cyclin complexes and Cdc25 family members drive cell cycle transitions.

The cyclin G1 and the cyclin G2 are new members of this family (Tamura et al., 1993). Both this cyclins are closely related to the Cyclin I and A (Horne et al., 1996). The cyclin G1 is involved in G2/M arrest of the cell cycle replying to DNA damage (Okamoto and Prives, 1999, Kimura et al., 2001). The cyclin G2 is a negative regulator of the G1/S transition (Bennin et al., 2002; Horne et al., 1997). Cyclin G1 is one of the target gene of the transcription factor p53 (Okamoto and Beach, 1994; Zauberman et al., 1995), the tumor suppressor implicated in the regulation of cell cycle, apoptosis, DNA repair, Cell differentiation (Barak et al., 1993; Wu et al., 1994). The cyclin G1 and G2 are the unique among the cyclin family that interact with the phosphatase PP2A (Bennin et al., 2002; Okamoto et al., 2002; Okamoto et al., 1996). It was also demonstrated the association of Cyclin G1 with cdk5 *in vivo* in mice (Kimura et al., 2001).

D. melanogaster has only one Cyclin G (Salving et al., 2008; Gildea et al., 2000) expressed ubiquitously during the development. The silencing of Cyclin G by RNA interference showed a high lethality demonstrating that this gene is essential for this stage. The Cyclin domain of Drosophila Cyclin G is very similar to the vertebrate Cyclin G1 and G2, showing and identity of 42% and 46% respectively. Than it was demonstrated that Cyclin G interacts with Widerborst (WDB), the regulatory subunit of PP2A such as with different Cdks including Cdk2 and Cdk4 (Giot et al., 2003; Stanyon et al., 2004).

3.3. Cross-talk between stress response pathways

Increasing evidence showed that different stresses response pathways evoked by stresses are regulate independently as well as co-ordinately, as a network of interlinked pathways (de Pomerai *et al.*, 2008; Kourtis and Tavernarakis, 2011), (Fig. 3.10.).

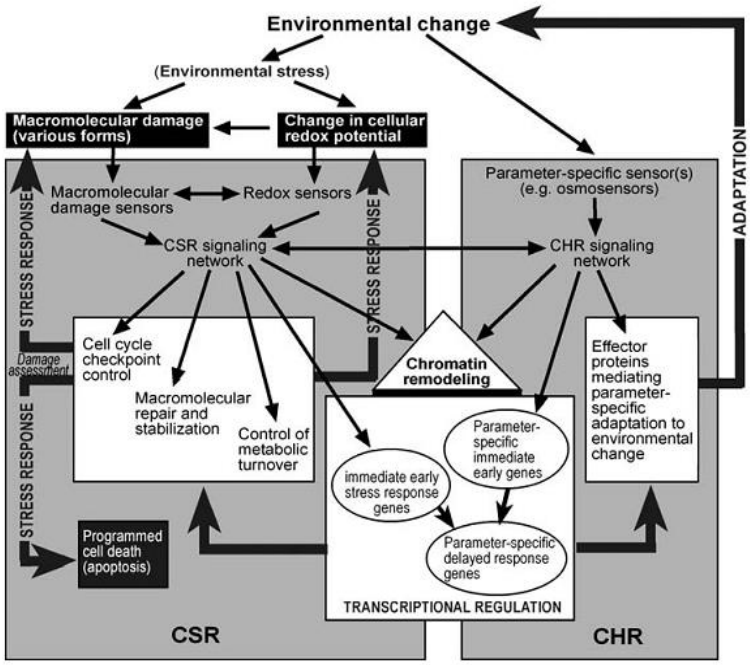

Fig. 3.10. Schematic representation of key aspects of the cellular stress response (CSR) and its interaction with the cellular homeostasis response (CHR). The CSR serves to restore macromolecular integrity and redox potential that are disturbed as a result of stress. In contrast, the CHR serves to restore cellular homeostasis with regard to the particular environmental variable that has changed. Both types of cellular responses to environmental change are interconnected at numerous levels (Kültz et al., 2005).

Organisms respond to endogenous metabolic requests as to many exogenous stresses as heat, cold, chemical toxicants ionizing radiation, primarily at cellular level, the fundamental unit of biological organization, through cellular stress response (CSR) through the cellular stress response pathways to limit cellular damage maintaining or re-establishing the cellular homeostasis (Simmons et al., 2009).

The CSR is considered the "guardian" of cellular homeostasis, it is more complex and involves many proteins (Kültz et al., 2005) to restore the homeostasis. Afterward it is important identify the link proteins that coordinate and regulate this cross- talk (Amanda et al., 2009).

Nowadays many genes involved in the CSR have been identified, but a lot of gaps remain to be clarify for their precise functions and interaction with other genes of CSR pathways (Kültz et al., 2003).

3.4. *Wolbachia* host manipulation on many pathways

Wolbachia are intracellular bacteria belonging to the order of Rickettsiales, comprising eight different supergroup (A-H) (Casiraghi et al., 2005) with mutualistic, commensal and parasitic relationship with the host. It is the most widespread endosymbiotic maternally transmitted α-proteobacteria found in arthropods infecting the 76% of all insect species (Werren et al., 2008). In nature, the *Wolbachia* infection is not perfect, than these bacteria have adopted same mechanisms to manipulate host reproduction as male killing, male feminization, parthenogenesis and cytoplasmic incompatibility (Hoffman et al., 1998; Bourtzis et al., 1998; Poinsot et al., 2003; Bossan et al., 2011).

It was demonstrated that *Wolbachia* infection influences the expression of genes involved in metabolism, immunity and reproduction in *D. melanogaster* (Zheng et al., 2011) (Fig 3.11.).

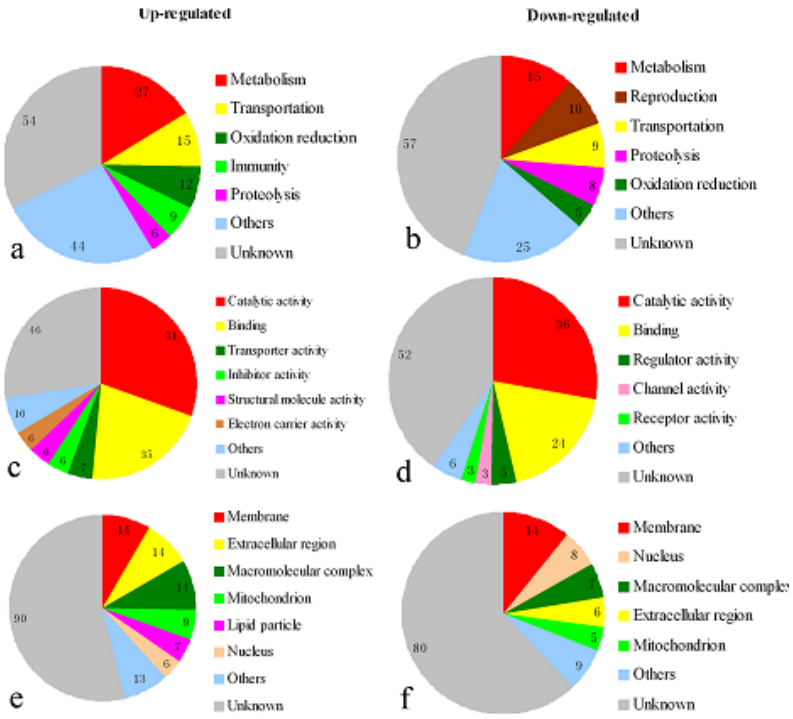

Fig. 3.11. Pie chart representation of gene ontology for genes differentially expressed in microarray analyses according to biological process (a, b), molecular function (c, d) and cellular component (e, f). Gene expression in Wolbachia-infected larval testes was compared to uninfected larval testes of Drosophila melanogaster and the criterion for differential expression was ≥1.5 fold changes with a q-value of <5% (Zheng et al., 2011)

Despite the great importance of the interaction between *Wolbachia* and cell symbiosis, the influence of *Wolbachia* on cellular stress response pathways has not yet been investigated. Moreover the infection is responsible of the reduction of sperm and eggs production (Snook et al., 2000; Hoffman et al., 1990). Some *Wolbachia* strain cause lesser eggs deposition and short life (Fry et al., 2002).

Wolbachia is also found in nematodes many of which are pathogenic for humans. These nematodes have an obligate relationship with *Wolbachia* and its removal causes the

nematodes development inhibition and death of the host (Rao et al., 2002; Chirgwin et al., 2003; Volkmann et al., 2003). Recently, emerged the capability of *Wolbachia* infection to inhibit the Dengue virus in *Aedes aegypti* (Fig. 3.12.).

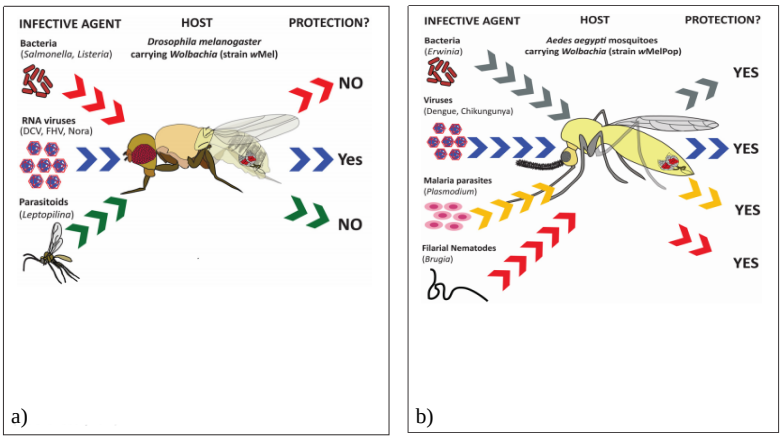

Fig 3.12. a) Effect of *Wolbachia* endosymbiotic bacteria on the ability of *Drosophila* to resist infection. Recent studies have shown that the presence of *Wolbachia* strain *w*Mel in *Drosophila melanogaster* confers resistance to infection by various RNA viruses (*Drosophila* C Virus, Flock House Virus, and Nora virus) (Teixeira et al., 2008), but not by intracellular bacterial pathogens (*Salmonella typhimurium* and *Listeria monocytogenes*) (Rottschaefer and Lazzaro, 2012) or parasitoid wasps (*Leptopilina boulardi*)(Martinez et al., 2012).**b) Influence of *Wolbachia* endosymbionts on inhibition/reduction in transmission capacity as well as protection of mosquitoes against infection.** *Aedes aegypti* mosquitoes transinfected with the *Wolbachia* strain *w*MelPop are protected from infection by pathogenic bacteria (*Erwinia carotovora*) (Kambris et al., 2009), viruses (dengue and Chikungunya) (Moreira et al., 2009), malaria parasites (*Plasmodium gallinaceum*) (Moreira et al., 2009), and parasitic filarial nematodes (*Brugia pahangi*) (Kambris et al., 2009) (Eleftherianos et al. 2013).

It is a promise in the field of the pest and of the disease vector control. (Hoffmann et al., 2011, Walker et al., 2011). Hence, despite the great importance of the interaction between *Wolbachia* and cell symbiosis, the influence of *Wolbachia* on cellular stress response pathways has not yet been investigated.

3.5. Health and environmental implications

Temperature is a physical variable that influences the distribution, the abundance of all specie (Cossins and Bowler, 1987). Always the environmental changes have influenced the evolution of life. In addition, the human activities continue to threaten the health of the biosphere with heavy impact on natural ecosystems.

From the ecological point of view, all trophic levels from plant to insect were affected by climatic change causing the destabilization of population dynamics that can lead to the extinction of the organism (van der Putten, 2004). As regards ectotherms, the temperature affects their distribution, the survival, the development and the growth, in addition to physiological and metabolic processes (Sinclair et al., 2003).

Therefore, it is important to better understand the mechanism of cells and organisms adaptation to environmental stress (Kültz et al., 2003), and to investigate on the mechanisms of stress response to cope to environmental health problems, to allow the basis for toxicological risk assessment, and to use bioindication processes monitoring the global environmental change (Kültz et al., 2005).

Moreover the study on cellular stress response, one of the most conserved pathway between all organisms, can lead implication on health problems elucidating the involvement of candidates' genes and proteins involved in the CSR,

because many disease are caused by dysfunction of the normal function of pathway.

3.6. Model organism

The Insecta are a class belong to the phylum of Arthropoda that is the greatest of all animal group in the word that constitute the 5/6 of all animal kingdom. The *Drosophila melanogaster* Meigen 1830, also know fruit fly, belonging to the family of Drosophilidae. In *D. melanogaster* was discovered for the first time the gene structure by the Nobel Prize Lewis in 1994; Weischaus and Nusslein-Volhard discovered many genes implicated in genetic development and embryogenesis. Moreover the *D. melanogaster* has its genome sequenced. It is constituted of about 14000 genes on four chromosomes: three autosomal chromosomes and the XY sex chromosomes in which the Y determine the male sex.

Otherwise its nucleotides and proteins sequence shows an identity of 40%, while the functional domain arrive at 80-90% of shared identity with mammals. All these reason make the *D. melanogaster* the model organism most commonly used in different research area considering the brief live cycle that pass from the eggs, the larva, the pupa, and the adult stages in 14 days at 25 °C; its low cost maintenance, and its

high productivity also. In fact a single mate can produce 400 hundred of eggs (Pandey and Nichols, 2011).To study the cellular stress response (CSR) the ideal model organism choosed is the fruit fly, for its small set stress response gene translated in a more simple CSR network (de Pomerai et al., 2008).

4. Aims

The research project carried out during my PhD thesis was focused on the activation of cellular stress response (CSR) after cold stress in *Drosophila melanogaster*, by monitoring the expression levels of Heat-shock-protein-70Aa (Hsp70Aa), Autophagy related gene-1 (Atg1), X-binding protein-1 (Xbp1) genes, respectively involved in the heat shock response, in the autophagy and in the endoplasmatic reticulum unfolded protein response. In particular, we focused on the effect of Cyclin G (CycG) and cyclin-dependent kinase 5 (cdk5) in the transcriptional activation of Hsp70Aa after cold stress. Moreover to study the possible involvement of *Wolbachia* infection on the CSR in the infected insects and to investigate also on the effects of the cold stress on *Wolbachia* infection, we monitored *Wolbachia* surface protein (wsp) gene expression upon different protocols of cold stress and recovery periods.

5. Materials and methods

5.1. Fly culture

Drosophila melanogaster Oregon R-C, a *Wolbachia* infected wild-type line, was obtained from Bloomington *Drosophila* Stock center, Indiana University. Flies were reared in 42 ml bottle at 25 °C a 12/12 h light-dark cycle. Standard medium was prepared with sugar (1.6%), yeast (3.2% w/v) and agar (3.2%) as just indicated by Colinet et al., 2009, with cornmeal (1.6%) and 2.5 g/l of Methyl 4-hydroxybenzoate.

5.2. Antibiotic treatment

To obtain genetically identical *Wolbachia* free *D. melanogaster* line (W^-) from the *Wolbachia D. melanogaster* infected stock (W^+), a pool of males and females flies were isolated on standard medium to which was added 0.25 mg/ml tetracycline antibiotic (Fry et al., 2004). The treatment was carried out for two generations.

The obtained *D. melanogaster Wolbachia*-free line was then maintained on standard medium to ensure the full recovery of the flies after the antibiotic treatment (Fry et al., 2004).

5.3. DNA extraction

To confirm that *Wolbachia* was cleared from treated *D. melanogaster,* DNA was extracted as described by Bouneb et al., 2014.

Briefly, for the DNA extraction 4 days old virgin females treated with antibiotic and untreated, were individually homogenized with a pestle in a 1.5 mL tube containing 100 µL of 6% InstaGene™ Matrix (Bio-Rad) and 10 µL of Proteinase K solution 20 mg/ml (5 PRIME). Samples were incubated at 56 °C for 30 min then vortexed at high speed for 10 s and boiled at 100 °C for 8 min. After the samples were centrifuged at 13000 rpm for 3 min. 5 µL of the supernatant was used to perform the wsp gene amplification, a gene coding for a protein surface of *Wolbachia* using wsp primers indicated by Braig et al., 1998.

5.4. RNA interference

RNA interference (RNAi) mediates gene silencing (Fire et al., 1998) through the formation of dsRNA-specific (dsRNAs) transformed to 21-23 bp dsRNAs called siRNAs through an enzyme named DICER (Bernstain et al., 2001). Finally the siRNAs mediates the degradation of complementary mRNA (Zamore et al., 2000) resulting in the knockdown of gene expression.

The GAL4/UAS system is commonly used for the analyses gain of function phenotypes. Combining this method with the RNA interference (RNAi) technique, became a powerful tool to study loss of function phenotypes (Duffy et al., 2002) to inactivate gene expression.

The Bloomington Stock center has a big Flies list that express GAL4 driver. The GAL4 drivers regulates gene transcription through the binding of Upstream Activating Sequences (UAS) elements (Duffy et al., 2002). In *D. melanogaster* was demonstrated that GAL4 regulates the expression of the gene reporter under UAS control (Fisher et al., 1988). The GAL4 and UAS sequences are present in two different lines and the silencing of the target gene became feasible after the cross of GAL4 driver line and UAS line that containing the target gene, in the progeny.

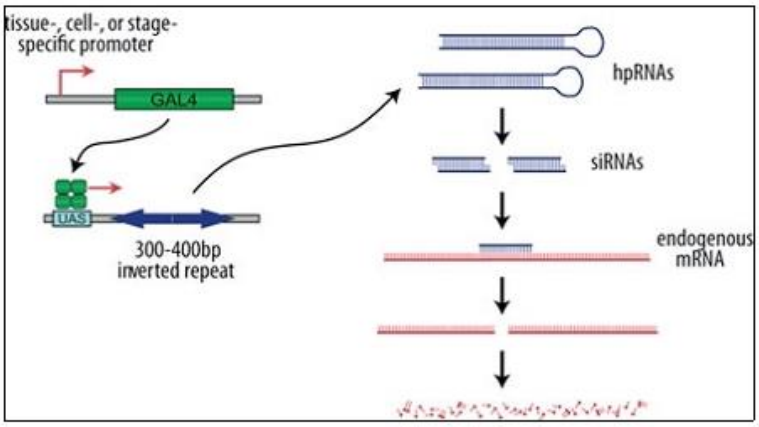

Fig. 5.1. RNAi mediated by GAL4/UAS system: GAL4/UAS was used to produces an hairpin RNA that was processed by DICER enzyme becoming siRNAs that evokes the degradation of a specific mRNA target.

Few studies used RNA interference (RNAi) to understand how genes respond to cold stress (Rinehart et al., 2007).

To study the possible involvement of the cdk5.gene in the activation of stress response by cold stress, the cdk5 was silenced trough RNAi techique using the GAL4/UAS system (Brand and Perrimon, 1993). Two UAS-cdk5 lines were obtained from Bloomington, Indiana University (ID 27517; ID 35287). Daughterless GAL4 line (coming from laboratory of prof. Giordano, department of Genetics, Federico II, Naples) was used as driver line resulting in general down-regulation of cdk5 gene. UAS females of both lines were crossed with GAL4 males. The F_1 was tested to verify the knockdown of cdk5.

5.5. Cold stress and recovery treatment

Cold stress treatments were performed on three groups, each one of 20 individuals of synchronized 4 days old virgin flies of both sex. They were sexed without CO_2 anesthesia because it can influence the stress recovery (Nilson et al., 2006). To induce the chill coma, the cold stress treatments were performed at 0° C in CH-100 heating/cooling block (Biosan) using 0.5 ml tubes immersed in 10% of glycol solution precooled. Knowing the timing of cold stress in which there is not flies mortality (Colinet et al., 2009), it were considered: 30 min (S30), 2h (S2h), 4h (S4h), 6h (S6h), 9h (S9h) of stress at 0

°C. After the cold stress flies were divided in two group: a group was recovered at 25 °C with standard food (RF) for: 30 min (R30), 2h (R2h), 4h (R4h), 6h (R6h), 8h (R8h) while another group was recovered without food (RA) for the same time, in medium containing only 1% agar as water source. The recovery phases were evaluated in presence or in absence of food to exclude any effect of deprivation of food on genes expression. For every time there was a corresponding control at 25 °C. After the treatments flies were transferred to 2 ml cryogen tubes and snap-frozen in liquid nitrogen ad stored at -80 ° C until RNA and protein extraction.

5.6. RNA extraction

RNA extraction was performed using the PureLink® RNA Mini Kit (Life technologies™) following the manufactures protocol. Every sample was:

- Homogenized on ice in fresh lysis buffer with 1% of 2-mercaptoethanol with a pestle in a 1.5 ml tube.
- Centrifugated at 2,600 x g for 5 min at room temperature (RT).
- The supernatant was transferred to a fresh RNAse free tube.

1. 0.5 volume 96-100% ethanol was added to each volume of tissue homogenate.
2. Mixed through shaking to disperse any visible precipitate that may form after the addiction of ethanol.

3. Up to 700 µl were transferred to a Spin Cartridge with the Collection Tube.

4. Centrifuged at 12,000 x g for 2 min at RT. Discarded the flow-through, and reinserted the spin cartridge in the same Collection Tube.

5. Repeated the step 3-4 until the entire sample was processed.

6. 700 µl of Wash Buffer I was added to the Spin Cartridge. Centrifuged at 12,000 x g for 15 sec at RT. The flow-through was discarded with the Collection Tube. The Spin Cartridge was placed in a new Collection Tube.

7. 500 µl of Wash Buffer II with ethanol (300 ml of 96% of ethanol were added when the Wash Buffer II was opened for the first time) was added to the Spin Cartridge.

8. Centrifuged at 12,000 x g for 2 min at RT. The flow-through was discarded and the Spin Cartridge was reinserted in the same Collection Tube.

9. The 7-8 steps were repeated once.

10. The Spin Cartridge was centrifuged with the Collection Tubes at 12,000 x g for 1 min at RT to dry the membrane with attached RNA. The Collection Tube was discarded. The Spin Cartridge was inserted into a Recovery tube.

11. 20 µl of RNase-Free Water was added to the center of Spin Cartridge and incubated for 2 min at RT.

13. The Spin Cartridge was centrifuged at 12,000 x g for 2 min at RT.

14. RNA extracted was stored or just processed with DNase treatment.

5.7. DNase treatment

Each sample was processed by TURBO DNA-free™ Kit (Life technologies) following the manufacture instructions.

1. 0.1 volume of 10X TURBO DNase Buffer and 1 µl TURBO DNase was added to the RNA and mixed gently.

2. Sample was incubated in a termoblock at 37 °C for 30 min.

3. 0.1 volume of DNase inactivation Reagent was added and mixed very well.

4. Sample was mixed occasionally during the 5 min of incubation at RT.

5. Sample was centrifuged at 12,000 for 2 min and RNA was transferred to a fresh tube avoiding to uptake the inactivation buffer on the bottom of the tube.

RNA purified was quantized by Qubit® 2.0 fluorometr with Qubit® RNA Assay kit. For each quantification it was prepared a Qubit™ Working Solution with 1 µl of Qubit™ Reagent in addition to 199 µl of Qubit™ Buffer. It were used 10 µl of each Standards provided by kit and 190 µl of the working solution to build the calibration line. 1 µl of every sample and 199 µl of

Qubit™ Working Solution were used for sample quantification, mixed for 3 s and after 2 min it was proceed to the RNA quantification.

5.8. Reverse transcription and PCRs reactions

300 ng of total RNA was used for the reverse transcription using SuperScript® VILO™ cDNA Synthesis Kit following the manufacturer's protocol.

1. For every reaction, were combined the following components in a tube on ice. For multiple reaction were prepared a master mix without RNA.

- 5X VILO™ Reaction Mix
- 10X SuperScript® Enzyme Mix
- RNA (up to 2.5 µg)
- DEPC-treated water

2. The tube contents were mixed gently and incubated at 25 °C for 10 min.
3. Follow an incubation at 60 °C of 60 min.
4. The reaction terminate at 85 °C for 5 min.
5. cDNA was stored at -20 °C until use and diluted 10 fold for the PCR and quantitative PCR (qRT- PCR) .

The coding sequences of Cyclin G (cycG); cyclin- dependent kinase 5 (cdk5); Autophagy-related gene-1 (Atg1), Heat shock factor (Hsf) and X box binding protein-1 (Xbp1), were retrieved

from Flybase (http://flybase.org/). The CycG and the Hsf genes showed multiple transcripts so only the common sequences were considered for the primers design. The primers for the Heat-shock-protein-70Aa (Hsp70Aa) and Ribosomal protein S20 (Rps20) were the same of Colinet et al., 2009. All primers sequences are reported in table 1.

Primer name	Primers Sequences (5'⟶3')	Temperature of melting [°C]
CycG_F	GCCCGATCAACCGCTTCTCC	63.5
CycG_R	GGGTGAAGTGGGCCAGTCC	63.1
cdk5_F	GCCTCAACGGGGAGATCGACA	63.7
cdk5_R	GGTTCTGTGGTTTCAGATCGCG	62.1
Hsf_F	CGATGCCGATACCAATCGCTT	59.8
Hsf_R	AGCTGGCCATGTTGTTGTGC	59.4
Hsp70Aa_F	TCGATGGTACTGACCAAGATGAAGG	63.0
Hsp70Aa_R	GAGTCGTTGAAGTAGGCTGGAACTG	64.6
Rps20_F	CCGCATCACCCTGACATCC	61.0
Rps20_R	TGGTGATGCGAAGGGTCTTG	59.4
Wsp_F	TGGTCCAATAAGTGATGAAGAAAC	52.3
Wsp_R	AAAAATTAAACGCTACTCCA	43.6
Xbp1_F	GGATGACGATAACATGGCTG	57.3
Xbp1_R	TCTCGTAGTCCATCTCCTCCAT	60.3
Atg1_F	GGGTTTGCGCGATTCCTG	58.2

Atg1_R	GCCAGGTTAGCATTCTGC	56.0

Table 1. Primers pairs used for PCR and qRT-PCR.

Reaction volume of PCRs is 25 µl. It contain: 1X PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, Primer for 0.3 µM, Primer rev 0.3 µM, Taq DNA polymerase (Invitrogen) 1.5 U, 5 µl of cDNA, H_20 to 25 µl. PCRs conditions adopted for the CycG, the cdk5, the Hsf and Hsp70Aa genes, were the follow: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 61 °C for 45 s, 72 °C for 30 s, finally a post amplification extension at 72 °C for 5 min. PCRs conditions for the Xbp1 and Atg1 genes amplifications were: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 57 °C for 50 s, 72 °C for 50 s, finally a post amplification extension at 72 °C for 7 min. For the amplification of wsp gene the PCR program was: 94 °C for 5 min, followed by 35 cycles of 94 °C for 1 min, 54,1 °C for 50 s, 72 °C for 50 s, finally a post amplification extension at 72 °C for 5 min.

PCR products were loaded in 2% agarose gel with 1X SYBR® Safe in DMSO (Invitrogen™), viewed and photographed on Gel Doc™ Bio-Rad to verify the PCRs amplifications.

Both DNA strands of PCRs products were sequenced at the 'Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale' (CIBIACI), Università degli Studi di Firenze, Italy. Sequence similarity searches were performed

using GenBank BLASTn http://blast.ncbi.nlm.nih.gov/Blast. All the amplification products obtained correspond to the expected sequences.

5.9. Real time PCR

To study the Atg1, Xbp1, Hsp70Aa, CycG, cdk5 and Hsf genes expression after different times of cold stress and recovery and to monitor the Hsp70Aa gene expression after the silencing of cdk5, Real time-PCRs (qRT-PCR) were carried out on LightCycler® 480 system (Roche Diagnostic) in 96 well plate (Applied Biosystems®, Life Technologies) in reaction volume of 20 µl containing: 10 µl of Power SYBR Master mix (Applied Biosystems, Life Technologies), 0.3 µM of each primer and 1 µl of cDNA diluted 10 fold. The qRT-PCR reactions conditions were 10 min at 95 °C followed by 39 cycle of 95 °C for 15 s, 56 °C for 30 s, 60 °C for 30 s followed by 1 cycle of 95 °C for 15 s, 60 °C for 1 min and 95 °C for 15 s, for melting curve analysis, to confirm the specificity of each primers pair. The relative quantification was evaluated using the $2^{-\Delta\Delta Ct}$ calculation method (Livak and Schmittgen, 2001) to evaluate quantitative variations. All qRT-PCR were evaluated by statistical analyses.

5.10. Statistical Analyses

One-way ANOVA was applied:

1) To evaluate the effect of *Wolbachia* on the CSR evaluating the fold change in gene expression of Xbp1, Atg1 and Hsp70Aa.

2) To study the timing gene expression of Hsp70Aa, Cyclin G, cdk5 and Hsf genes after different times of stress and recovery.

Post-hoc comparison (Tukey test, $P<0.05$) was performed to analyse the difference in gene expression between treatments. All statistical analyses were carried out with SPSS v.13.0 (SPSS, 2004).

T test was performed to evaluate the effect the influences of the food in Hsp70Aa, Cyclin G, cdk5 and Hsf genes expression in both sex ($P<0.05$) with SPSS v.13.0 (SPSS, 2004).

5.11. Protein extraction

Protein extraction was carried out using the Cell Extraction Buffer (Invitrogen™) (to see section 10.1. of supplementary materials for the buffer formulation).

To have the buffer ready to use, it is necessary to add 1 mM phenylmetthylsulphonyl fluoride (PMSF) (Delchimica) and 1X

Serine Protease Inhibitor Cocktail set I (Calbiochem). Each sample was:

- Lysed in the buffer fresh made for 30 min, on ice, vortexing every 10 min.
- Centrifuged at 13000 rpm for 10 min at 4 °C.
- The lysate was aliquoted and stored at - 80 °C.

Protein quantification was carried out by Qubit ® Protein Assay kit. For each quantification it was prepared a Qubit™ Working Solution with 1 µl of Qubit™ Reagent in addition to 199 µl of Qubit™ Buffer. It were used 10 µl of each Standards provided by kit and 190 µl of the working solution to build the calibration line. 1 µl of every sample and 199 µl of Qubit™ Working Solution were used for sample quantification, mixed for 3 s and after 15 min it was proceed to the proteins quantification. As calibrator was used the extraction buffer.

5.12. Co-immunoprecipitation

The protocol of Co-immunoprecipitation (Co-IP) allow to study proteins interaction using an antibody for the protein of interest. In this way it is possible to isolate the protein recognize by the specific antibody and the proteins associated. In this case, it want to study the possible interaction between the cdk5 and cycG. The protocol adopted for the Co-immunoprecipitation

(Co-IP) is just described by Cernilogar et al., 2011, briefly discussed as follow.

Before starting it was prepared:

- The TEA_{150} buffer (to see the 10.3. section of supplementary materials for preparation details).

- A mix containing 80 µl of Dynabeads® Protein A (Life Technologies) and 80 µl of Dynabeads® Protein G (Life Technologies). The Dynabeads® mix was washed three times with 1 ml of TEA_{150} buffer. After the last wash, it was added 70 µl of TEA_{150} buffer at the Dynabeads® mix A/G and the total volume was divided in four parts: two parts of 30 µl for the pre-cleaning phase and two parts of 40 µl for the binding phase.

550 µg of total protein (extracted as indicated previously) were mixed with the TEA_{150} buffer up to volume of 1 ml. After the protein extracts were added to the two 30 µl of ready Dynabeads® mix, (one for the immunoprecipitation of Cyc G and one for the immunoprecipitation of cdk5) followed by an incubation at 4 °C for 1 h at 4 °C in rotation. This is known as pre-cleaning phase because purify the extract from proteins that have no specific interaction with the Co-IP component. Each cleared extract was transferred to 40 µl of Dynabeads® mix A/G at which it was added 5 µg of the specific antibody selected for the immunoprecipitation: the IgG_1 anti mouse cdk5 1 µg/µl, monoclonal antibody (Millipore™) and the IgG anti rabbit

Cyclin G 0.5 µg/µl, polyclonal antibody (abcam®). The extract was incubated over night at 4 °C in rotation. In this phase, the specific antibody bind with the Fab region the specific protein and with Fc region to the Dynabeads® mix A/G. Next day the supernatant was recovered and stored at -80 °C. Follows the Elution step. For this purpose, 20 µl of Sample Buffer 2X (to see the 10.2. section of supplementary materials for the 2X Sample Buffer preparation) were added to the Dynabeads® mix A/G complexed with antibody and proteins, incubated for 5 min at RT. The eluted sample was stored at -20 °C until SDS-PAGE analysis.

5.13. SDS-PAGE and Western Blotting

For the proteins expression analysis of Cyclin G (CycG) and cyclin dependent–kinase 5 (cdk5) after different time of recovery post cold stress, 20 µg of total protein extract were separated by SDS-PAGE using the XCell II™ Blot module (Life technologies™) than analyzed by Western Blot.

Samples prepared with 2X Sample buffer (just described above) were boiled at 100 °C for 10 min, centrifuged at 12000 rpm for 5 sec and loaded in NuPAGE® Bis-Tris precast mini gels (Life technologies™), containing 12% polyacrylamide, in 1X NuPAGE® MES running buffer (Life technologies™) adding 500 µl of NuPAGE® Antioxidant in the Upper Buffer Chamber. In

the gel was loaded 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Life technologies™) for every proteins electrophoresis. For the analyses of the co-IP extracts, 15 µl of each eluted samples were boiled at 100 °C than loaded in the NuPAGE® Bis-Tris precast mini gels (Life technologies™). 10 µl of SeeBlue® Plus2 Pre-Stained Standards (Life technologies™) were used for every electrophoresis.

Gels run condition were: 200V constant for 45 min.

Subsequently, the separated proteins were transferred to the nitrocellulose filter of 0.2 µm by iBlot® Dry Blot System (Invitrogen™).

- The efficiency of the transfer was confirmed staining the filter with Ponceau Red for 5 min. After the filters were de-stained with distilled water and incubated with the blocking solution containing: 1% non-fat milk in 1X Phosphate Buffer Saline (PBS) and 0.03% $tween_{20}$ for 1 hour at room temperature in constant rotation. Afterward the membranes were incubated with the specific primary antibodies. The primary antibody used are:
- -Rabbit monoclonal IgG anti RpS20 (Abcam®), diluted 1:1000, with cross reactivity for *D. melanogaster* RpS20.
- Mouse monoclonal IgG_1 anti cdk5 (Millipore™), diluted 1:500, with cross reactivity for *D. melanogaster* cdk5.
- Rabbit policlonal IgG anti CycG (Abcam®), diluted 1:500.

For this protein it was necessary multiple sequences alignment

between the GycG of different species searching the major homology region with *D. melanogaster* CycG.

All antibodies were incubate O/N at 4 °C.

Nitrocellulose membranes were washed three times for 5 min, in solution constituted by 1X PBS and 0.03% $tween_{20}$.

Follows the incubation for 2 h at room temperature, in rotation, with secondary antibodies: the Horse radish peroxidase (HRP) goat Anti Rabbit IgG (diluted 1:10000) recognizes CycG; the HRP-goat anti mouse IgG_1 (diluted 1:8000) recognizes cdk5;

Nitrocellulose membranes were washed three times also as described before.

The signal developed by HRP, conjugated at secondary antibody, was revealed by Novex® Chemioluminescent substrates (Invitrogen™).

Western blot results were analyzed and normalized by densitometry analyses converting the intensity bounds signal in histograms through the ImageJ program.

6. Results and discussion

My PhD thesis work started searching on FlyBase (http://flybase.org/) the coding sequences of the key genes involved in cellular stress response (CSR): X box binding protein-1 (Xbp1); Autophagy related gene-1 (Atg1); Heat-shock-protein-70Aa (Hsp70Aa). For Xbp1 and Atg1, I found multiple transcripts that were aligned using BioEdit tool. Only the common sequences were considered for primers design and the primer sequences were always spanning an exon-intron boundary. For Hsp70Aa gene and the housekeeping gene Ribosomal protein S20 (Rps20) I used primers described by Colinet et al., 2009. RT-PCR reactions were carried out also on genomic DNA extracted from *Drosophila melanogaster* to verify primers specificity. A negative control was included in the PCR reactions. RT-PCR products were analyzed in 2% agarose gel and sequenced at the Centro di Servizi per le Biotecnologie di Interesse Agrario Chimico ed Industriale (CIBIACI), Università degli Studi di Firenze, Italy.

The sequences obtained were manually checked using FinchTV 1.4.0 program in case of not clear sequences, and blast similarity search were performed using http://blast.ncbi.nlm.nih.gov/Blast. All sequences obtained corresponded to the cDNA sequences deposited in GenBank.

6.1. Cold stress affects Atg1, Xbp1, Hsp70Aa gene expression

To study the involvement of the Atg1 and Xbp1 genes, in addition to Hsp70Aa, in the cellular stress response after cold stress, four days old virgin *D. melanogaster* females were used for the cold stress protocol described by Colinet et al. (2009), followed by recovery phase at 25 °C for two hours (section 5.5.).

RNA extraction, purification and DNase treatment were performed as described in section 5.6. Three hundred nanograms of total RNA were used for cDNA synthesis. (sections 5.6-8.)

The qRT-PCR for Atg1, Xbp1, Hsp70Aa and RpS20 was carried out on cDNA from cold stressed and control females. A melting curve analysis was performed to confirm the specificity of each primers pair. Relative expression of genes were calculated through the $2^{-\Delta\Delta Ct}$ calculation method (Livak and Schmittgen, 2001) to evaluate quantitative variations. The cold stress treatment significantly affected gene expression for all the considered genes (ATG1: $F_{1, 3} = 27.37$, $P < 0.005$; Xbp1: $F_{1, 3} = 322.79$, $P < 0.005$; Hsp70Aa: $F_{1, 3} = 277.80$, $P < 0.005$). Atg1 and Hsp70Aa genes, were up-regulated respectively 1.90 and 4.1 fold, while Xbp1 was down regulated, 0.03 fold (Fig. 6.1).

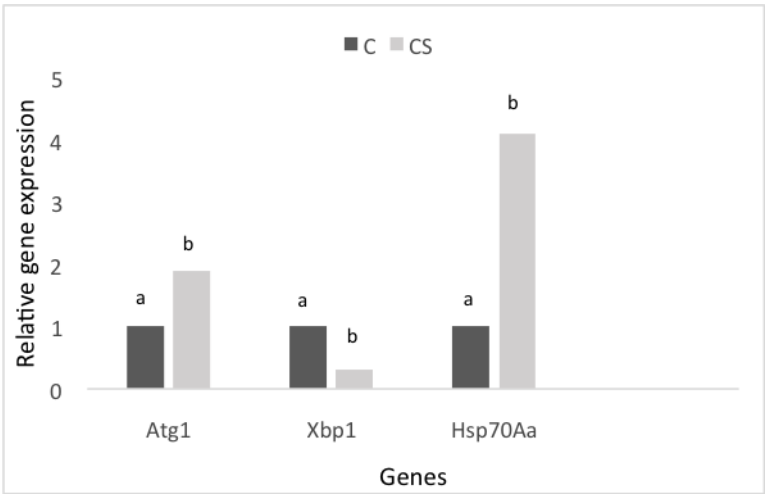

Fig. 6.1. Relative Atg1, Xbp1 and Hsp70Aa genes expression of cold stressed flies (CS) in comparison to control flies (C). Different letters between treatment indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; $P < 0.05$).

Hsp70Aa resulted to be the most responsive gene upon cold stress treatment amongst the studied genes, so it was established to focus the attention on the activation of the heat shock response by cold stress.

6.2. Cyclin G and cyclin-dependent kinase 5 involvement in the activation of the stress response by cold shock

It is known that the transcription of Hsp70 is regulated by the protein kinase C (PKC), a serine threonine kinase enzyme (Ding et al., 1998). However, increasing evidences indicate that numerous genetic pathways are involved in stress response (de Pomerai et al., 2008), not excluding that other serine/threonine kinase enzyme activate Hsp70 transcription. Therefore, it was studied the possible

involvement of the Cyclin G (CycG) and cyclin-dependent kinase 5 (cdk5) in the activation of stress response by cold stress. The transcripts of cdk5 and CycG and Hsf were retrieved in http://flybase.org/. For CycG and Hsf I found multiple transcripts, therefore only the shared regions were considered for primers design. Specific primers for the CycG, the cdk5 and the Hsf sequences were designed on regions spanning exon-intron boundaries and control RT-PCR reactions were performed also on genomic DNA (section 5.3.), from *D. melanogaster* females. RpS20 primers are the same described before. A negative control was included too. PCRs products were loaded in 2% agarose gel and sequenced.

The sequences obtained were checked and blast similarity search were performed with http://blast.ncbi.nlm.nih.gov/Blast. All sequences obtained corresponded to the genes sequences deposited in GenBank.

Afterward the primers' specify test, it was studied the influence of the sex and the food on the expression of GycG, the cdk5, Hsf and Hsp70Aa genes.

For this purpose, three groups of twenty flies (kindly donated by prof. Callaini, University of Siena) for both sexes (4 days old, virgin, reared at 25 °C) were considered. RNA extractions and cDNA synthesis (section 5.6-8.) were performed and the relative expression of CycG, cdk5, Hsf

and Hsp70Aa genes was evaluated. RpS20 was used as reference gene (section 5.9.). The relative quantification was evaluated using the $2^{-\Delta\Delta Ct}$ calculation method (Livak and Schmittgen, 2001). Data of genes expression showed no significant differences between males and females, recovered with food (wf) and without food (nf) (Fig. 6.2.).

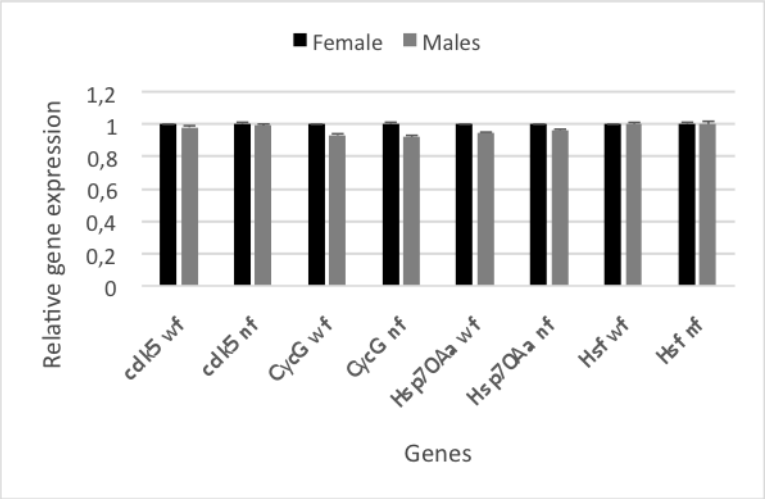

Fig. 6.2. Relative cdk5, CycG, Hsp70Aa and Hsf genes expression in presence of food (wf) or in absence of food (nf) in *D. melanogaster* females and males. There is no significance between the sex and the presence or absence of food difference in fold change (Student t test of independent samples; $P< 0.05$).

Student t test showed no significance between both sex and the presence or absence of food (male wf/nf: CycG t= 0.612, df= 4; cdk5 t=0.759, df= 4; Hsf t=1.512, df=4; Hsp70 t=0.480, df=4. Female/male wf: CycG t= 0.062, df= 4; cdk5 t=1.732, df= 4; df=4; Hsp70 t=4.330, df=4. For Hsf no sample variability. Female/male nf: CycG t= 6.928, df= 4; cdk5 t=0.822, df= 4; Hsp70 t=2.309, df=4; Hsf t= 1.512, df=4). Based on these observations I considered only females with normal food administration for the time-course analysis

of the CycG, the cdk5, the Hsf and Hsp70Aa genes expression.

6.2.1. Time-course analysis of CycG, cdk5, Hsf and Hsp70Aa genes expression

To study the temporal genes expression of Cyclin G and cdk5 in addition to Hsf and Hsp70Aa, qRT-PCR were performed using different protocols for cold stress and recovery.

With this aim, three biological replicate were considered, each one of 20 females, 4 days old. The protocols used for cold stress were consisting in different periods at 0 °C (section 5.5): 30 min (S30), 2h (S2h), 4h (S4h), 6h (S6h), 9h (S9h). After the cold stress flies were recovered at 25 °C with standard food (section 5.1) for different periods: 30 min (R30), 2h (R2h), 4h (R4h), 6h (R6h), 8h (R8h) after the time of stress established. For every time point there was a corresponding control at 25 °C. After the stress, flies were sacrified, RNA extracted, cDNA synthesized and qRT-PCR performed to evaluate gene expression. The relative quantification was evaluated. The treatments with different time for the cold shock showed significant effects on the expression all the considered genes excluding Hsf gene (CycG: $F_{5\text{-}12}$= 6.043, P= 0.004; cdk5: $F_{5\text{-}12}$= 6.961, P=0.003; Hsf: $F_{5\text{-}12}$= 1.463, P= 272; Hsp70Aa $F_{5\text{-}12}$= 17.524, P= 0.000).

Differently, the treatment with different recovery time, resulted in a significant change for the expression of all the genes (CycG: $F_{5\text{-}12}$= 24.562, P= 0.000; cdk5: $F_{5\text{-}12}$= 75.000, P=0.000; Hsf: $F_{5\text{-}12}$= 35.627, P= 0.000; Hsp70Aa $F_{5\text{-}12}$= 44.442, P= 0.000) (Fig. 6.3, 6.4, 6.5, 6.6).

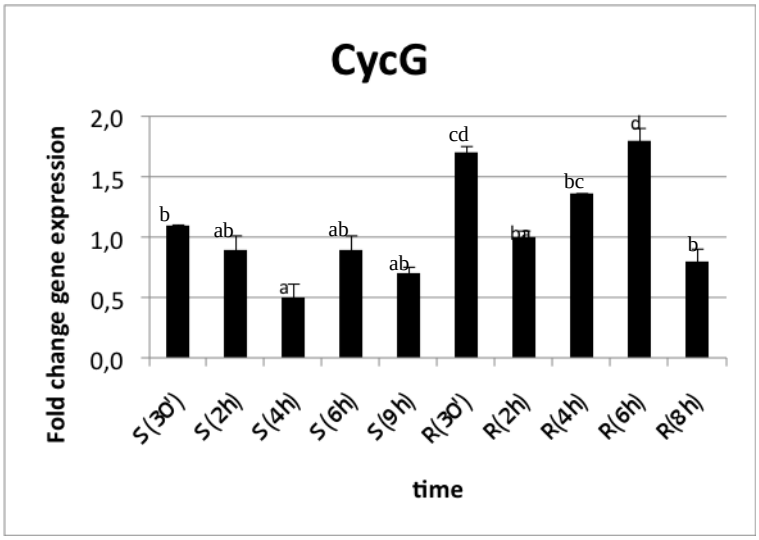

Fig. 6.3. Relative Cyclin G (CycG) gene expression after different times of stress (S) and recovery (R). Different letters between treatment indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; P< 0.05).

As shown in fig. 6.3, the CycG gene expression was lower during the cold stress period. The CycG expression reaches the maximum expression level after 30 min and 6 h of recovery.

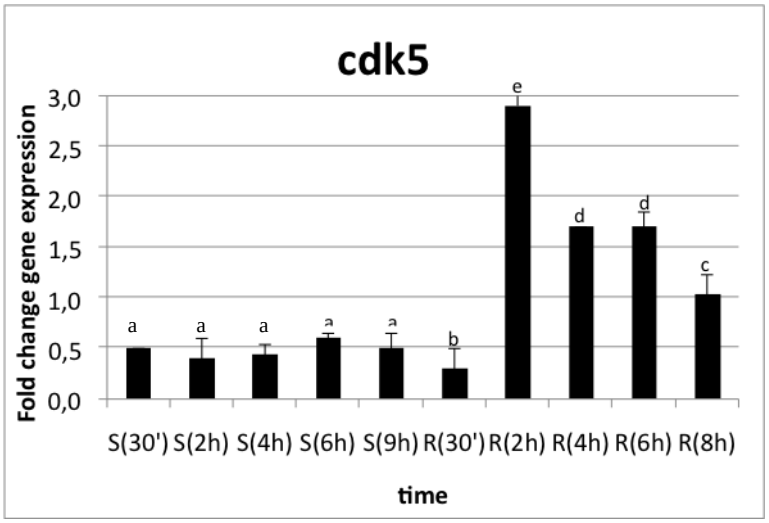

Fig. 6.4. Relative cyclin-dependent kinase 5 (cdk5) gene expression after different times of stress (S) and recovery (R). Different letters between treatment indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; P< 0.05).

As shown in fig. 6.4 the cdk5 gene expression is lower in the cold stress period than in the recovery time, showing the pick in gene expression after 2 h.

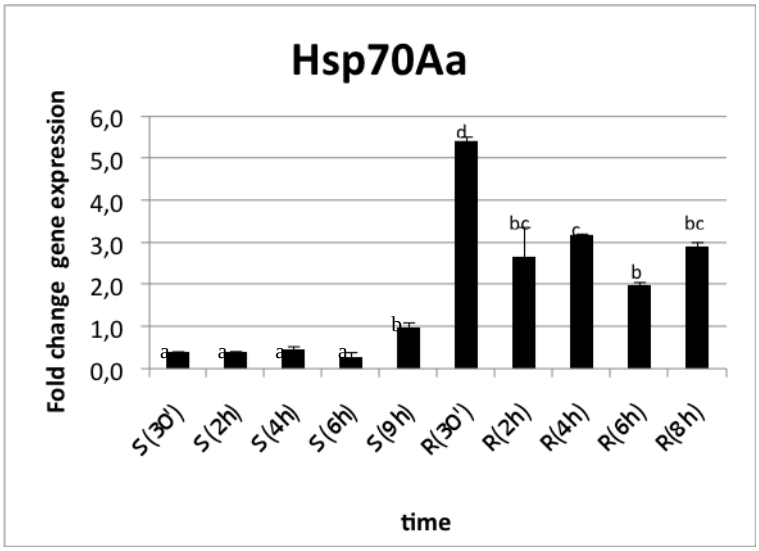

Fig. 6.5. Relative Heat-shock-protein-70Aa (Hsp70Aa) gene expression after different times of stress (S) and recovery (R). Different letters between treatment indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; P< 0.05).

As shown in fig. 6.5 the Hsp70Aa gene showed lower expression during the cold stress period compared to the recovery times. At the R30 time Hsp70 had the highest expression that remain high until 8h of recovery.

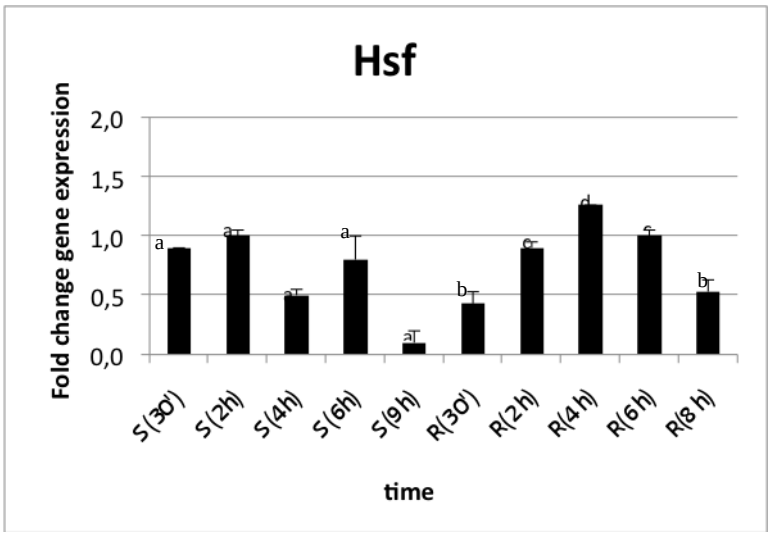

Fig. 6.6. Relative Heat shock factor (Hsf) gene expression after different times of stress (S) and recovery (R). Different letters between treatment indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; P< 0.05).

As shown in fig. 6.6 the change in Hsf gene expression was not significant during the cold stress period remaining lower during the recovery time. For this gene there is not data in literature at now after stress.

Overall, the obtained results were in accordance with previous reports (Colinet et al, 2009; Zhang et al, 2008). In fact, the studied genes were only activated during the recovery phase than during cold stress. Se scrivi così sminuisci il tuo lavoro perchè sembra che tutto ciò che hai

fatto è solo una conferma di quanto già pubblicato, scrivi meglio ☺

6.2.2. Timing protein expression of the CycG and cdk5

As known, in eukaryotic cells there are regulations in transcription and in translation. The study of proteins expression, in addition to gene expression, is important because proteins and their interaction are the causative forces in the cell (Greenbaum et al., 2003).

Before investigating on the cdk5 involvements in the activation of the heat shock response by cold, and on the interaction between the CycG and the cdk5 proteins, the expression profiles were evaluated after different times of recovery after cold stress considering that the expression change in recovery phases in comparison to stressing period. Three groups of twenty *D. melanogaster* females (4 days old, virgin) were considered. The recovery times considered ranged from 2 hours to 8 hours after 9 hours of stress. For every time there was a corresponding control at 25 °C. All samples were stored at -80 °C until analysis.

Hence, proteins extraction and quantification of all sampling times were performed (section 5.11.)

20 µg of total proteins extracts were loaded with LDS sample buffer in precast gel NuPAGE® Bis-Tris Mini gels containing 4-12% polyacrylamide (paragraph 5.13). Gel electrophoresis was carried out at 200 V constant for 45 minutes. After the separated proteins were transferred to nitrocellulose membrane of 0.2 µm through iBlot® Dry Blot System (Invitrogen™).The transfer efficiency was confirmed with a Ponceau Red staining (Fig. 6.6).

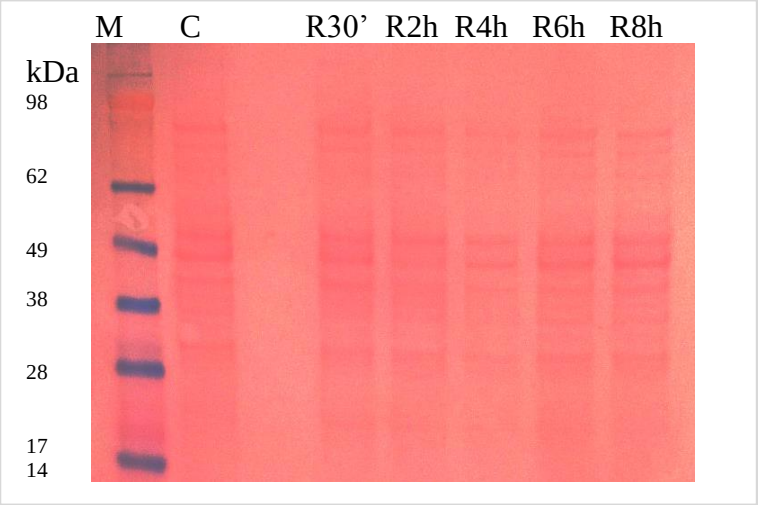

Fig.6.6. Total proteins extracts from stressed and recovered females at different times, transferred on nitrocellulose membrane 0.2 µm. M: 5 µL of SeeBlue® Plus2 Pre-Stained Standard marker (Life technologies), C:20 µg tot of controls females kept at 25°C, R30': 20 µg tot of cold stressed females at 0°C for 9 hours and recovered for 30 min, R2h: 20 µg tot of cold stressed females at 0°C for 9h and recovered for 2 hours, R4h: 20 µg tot of cold stressed females at 0°C for 9 hours and recovered for 4h, R6h: 20 µg tot of cold stressed females at 0°C for 9 hours and recovered for 6h, R8h: 20 µg tot of flies stressed for 9 hours at 0°C and recovered for 8 hours. All recovery time were made at 25 °C.

As shown in fig. 6.6, the concentrations of all total protein extracts are similar. Follows Immunoblotting (section 5.13) for timing proteins expression with antibody anti CycG an anti cdk5 (fig.6.7).

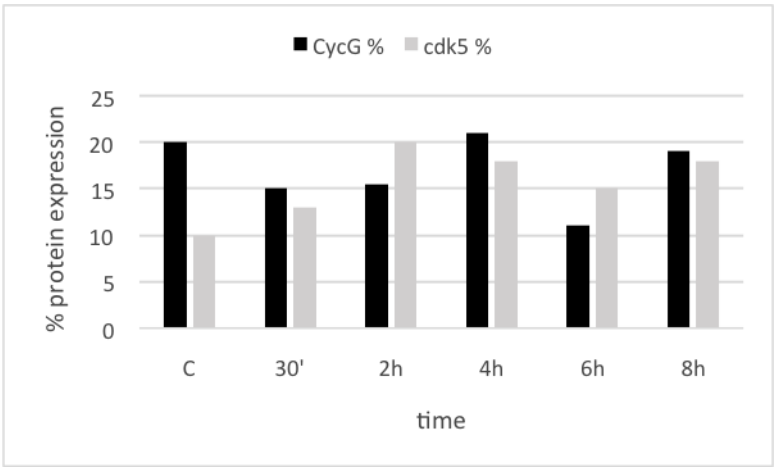

Fig.6.7. Densitometric analyses of the CycG and cdk5 immunoblotting normalized in comparison to RpS20 conducted by ImageJ program.

The Fig. 6.7. revealed the temporal Cyclin G and cdk5 proteins expression profiles. At the 2 h of recovery it was seen the highest expression of cdk5, while for the CycG is visible after 4 h of recovery.

6.3. Co-immunoprecipitation of Cycling-cdk5

The co-immunoprecipitation protocol was carried out to investigate on the Cyclin G and cdk5 interaction using protein extract after 30 min (R30') and (2h) of recovery and the control protein extract (C) . The immunoprecipitated extracts R30' and C with the antibody anti cyclin G and anti cdk5 (section 5.12.) were loaded in 4-12% polyacrilammide gels with 20 µg of the R30' and C extract not immunoprecipitated (input), used as controls. After the gel were transferred to nitrocellulose membrane of 0.2. µm.

Here, it was reported only the co-immunoprecipitation made with the cdk5 antibody (fig. 6.9.).

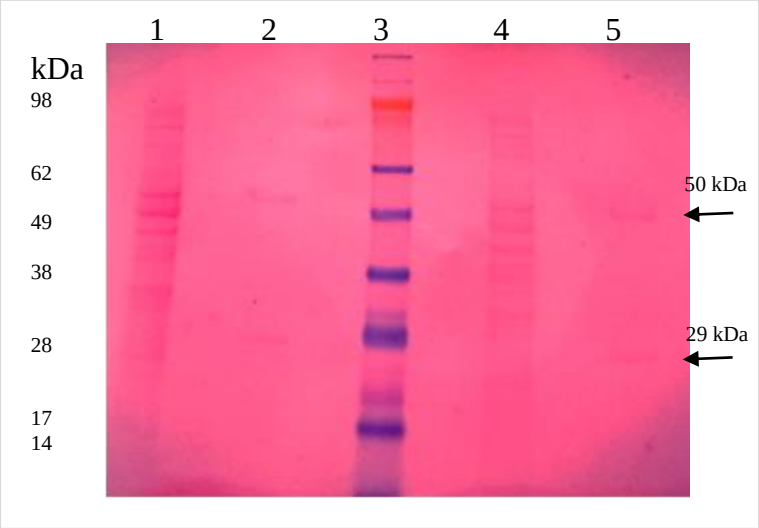

Fig.6.9. Total proteins extracts transferred on nitrocellulose membrane 0.2 µm from control flies (C) and flies stressed for 9 hours at 0°C and recovered for 30 min (R30') at 25°C. 1: 20 µg of total proteins extracts of input C, 2: 15µl of C immunoprecipitated extracts, 3: 5 µL of SeeBlue® Plus2 Pre-Stained Standard marker (Life technologies), 4: 20 µg of total proteins extracts of input R30', 5: 15µl of R30' immunoprecipitated

Red Ponceau staining showed that all the proteins concentration are similar for all samples. The two bands of 50 kDa and 29 kDa in second and fifth lanes of the fig. 6.9 corresponding to heavy and light antibody chains. Follow the indirect immunorilevation of Cyclin G on the membrane previously showed (fig. 6.10).

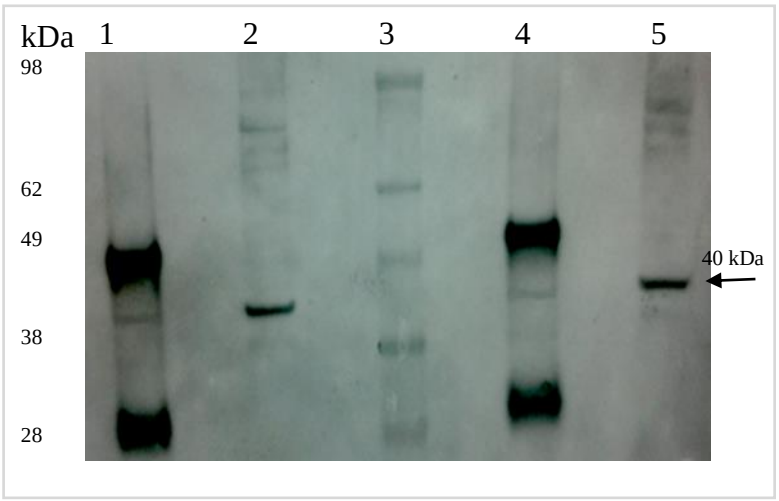

Fig. 6.10. Indirect immunorivelation on the membrane showed in fig.6.9 of Cyclin G on the C and R30' protein extract immunoprecipitated with cdk5 antibody.

In correspondence of the arrow is detected a band of 40kDa corresponding to the expected molecular weight of Cyclin G, in the immunoprecipitated proteins extracts on control flies, lane 1, and on the R30' immunoprecipitated proteins extracts, lane 4. The same band is visible in the respectively input sample, lane 2 for control and lane 5 for R30' extracts. Therefore this result demonstrate the CycG/cdk5 interaction.

Afterward it was done the direct immunorilevation of the cdk5 on the same filter used for the indirect imunorivelation of CycG to confirm this interaction (fig. 6.11).

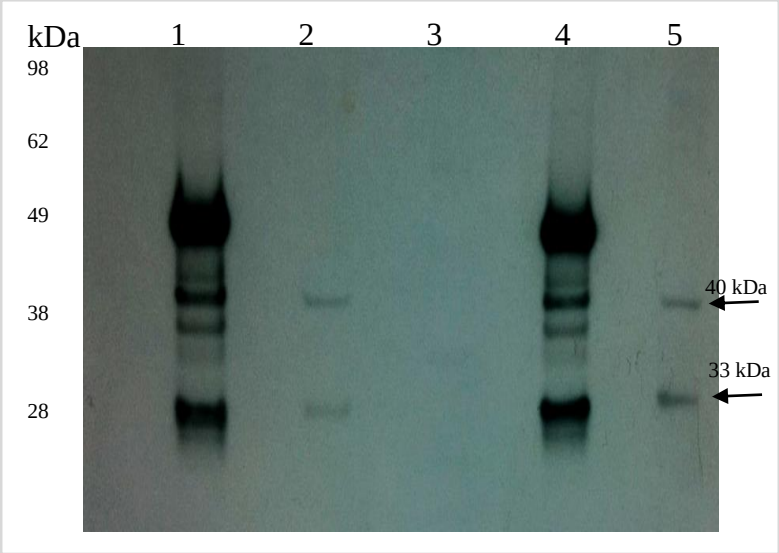

Fig. 6.11. Direct immunorivelation on the membrane showed in fig.6.9 of cdk5 on protein extract immunoprecipitated with cdk5 antibody.

The Western blot showed in fig 6.11 confirms that the CycG interact with cdk5, as indicated by the arrows in correspondence of 40kDa and 33kDa present in both inputs, lanes 2 and 5, as in the immunoprecipitated C and R30' samples, lanes 1 and 4.

Follow the SDS-PAGE in 4-12% polyacrilammide gels of the R2h and C immunoprecipitated extracts with the antibody anti cdk5 (section 5.12.) and their respective inputs. The gel was transferred to nitrocellulose membrane of 0.2. µm (fig 6.12).

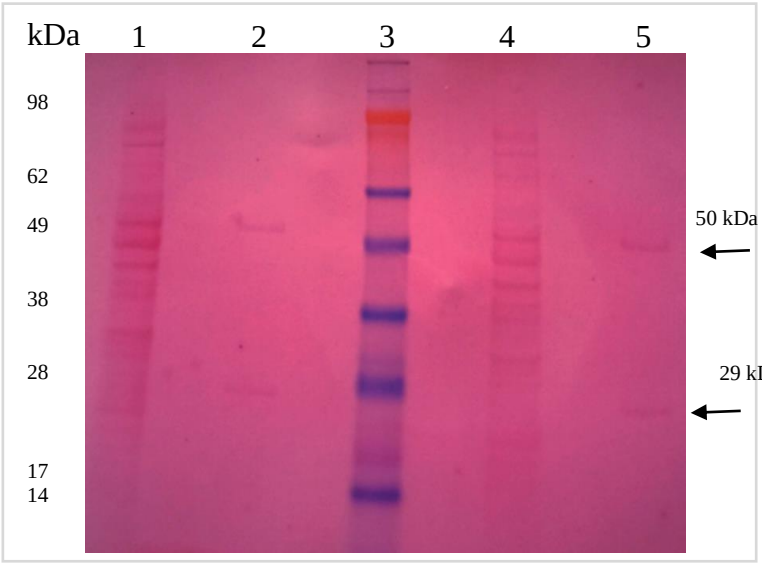

Fig.6.12. Total proteins extracts transferred on nitrocellulose membrane 0.2 µm from control flies (C) and flies stressed for 9 hours at 0°C and recovered for 2 hours (R2h) at 25°C. 1: 20 µg of total proteins extracts of input C, 2: 15µl of C immunoprecipitated extracts with antibody anti cdk5, 3: 5 µL of SeeBlue® Plus2 Pre-Stained Standard marker (Life technologies), 4: 20 µg of total proteins extracts of input S2h, 5: 15µl of S2h immunoprecipitated extracts with antibody anti cdk5 .

As showed in fig 6.12, all the proteins concentration are similar for all samples, the heavy and light antibody chains of 50 kDa and 29 kDa are visible in second and fifth lanes as the previous filter. Hence, the indirect immunorilevation of Cyclin G on (R30') and C proteins extracts immunoprecipitated with cdk5 was done (fig. 6.13)

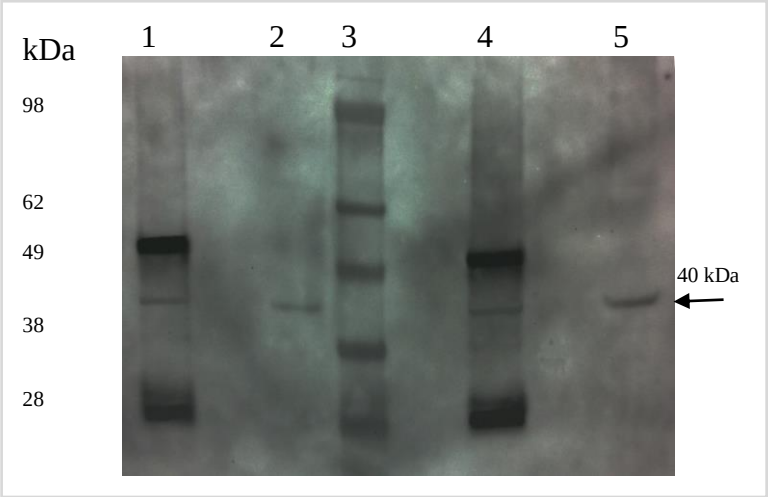

Fig. 6.13. Indirect immunorivelation on the membrane showed in fig.6.13 of CycG on the C and R2h proteins extracts immunoprecipitated with cdk5 antibody.

The figure 6.13 showed the same 40 kDa band of the fig. 6.10 corresponding to the expected pM of CycG in the C and R2h inputs as in the C and R2h immunoprecipitated samples with antibody anti cdk5. In this experiment it was seen that after two hours of recovery the interaction between these two proteins exists yet. Moreover the direct immunorilevation of the cdk5 on the filter showed in fig 6.12 using cdk5 antibody was done (6.14).

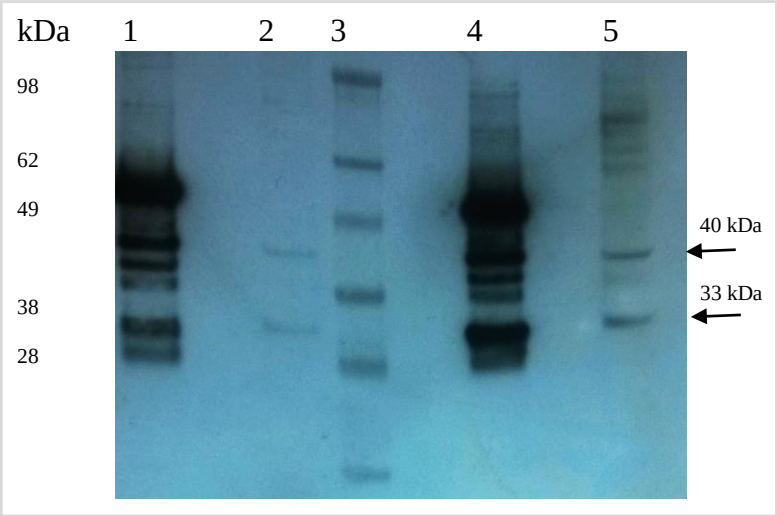

Fig. 6.14. Direct immunorivelation on the membrane showed in fig.6.13 of Cdk5 on C and S2h input proteins extracts and C and S2h immunoprecipitated proteins extracts with cdk5 antibody.

The figure 6.14 confirm the interaction between the CycG and Cdk5 after 2 hours of recovery post cold stress. In fact the 40 kDa and 33kDa corresponding to CycG and cdk5 are present in all samples immunoprecipitated or not as indicated by the arrow.

6.4. Hsp70Aa transcriptional regulation by cdk5 after cold stress

To investigate on possible involvement of the cdk5 in the activation of the heat shock response after cold stress, the cdk5 silencing was carried out by RNA interference (RNAi) using the GAL4/UAS system (see 5.4. section for details). The F_1 generation was tested to verify the cdk5 knock-down. qRT-PCR were carried out to control the cdk5 gene

expression on RNAi flies in comparison to wild type line treatment (Fig. 6.15-16).

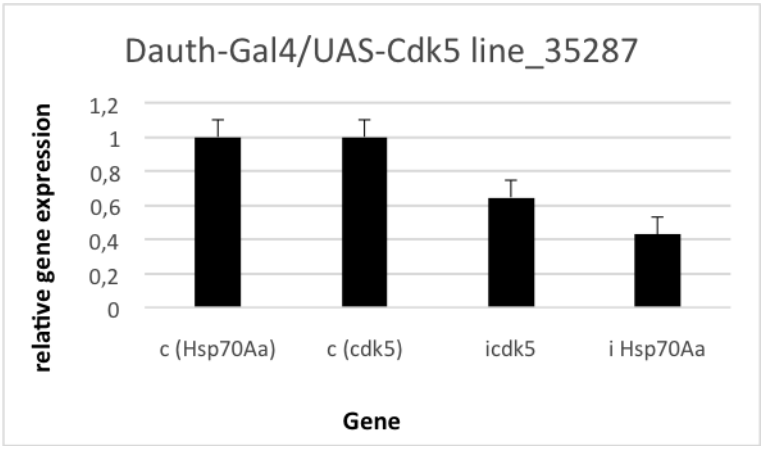

Fig.6.15. Fold change gene expression of Cdk5 and Hsp70Aa in control line and silenced Dauth-Gal4/UAS-Cdk5 line_35287.

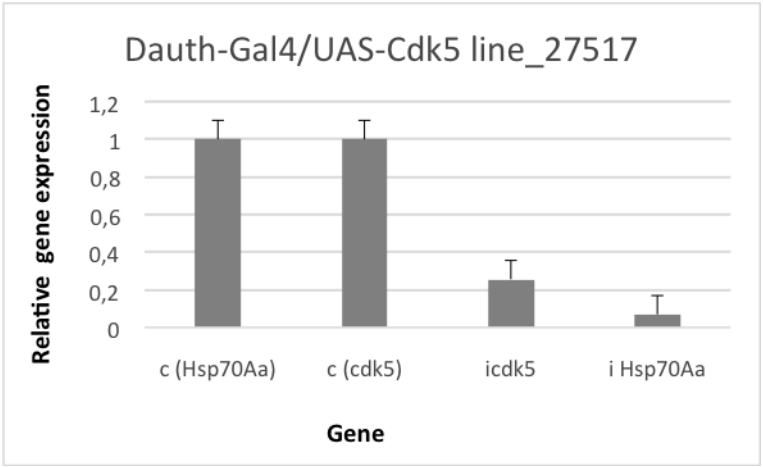

Fig.6.16. Fold change gene expression of Cdk5 and Hsp70Aa in control line and silenced Dauth-Gal4/UAS-Cdk5 line_27517.

As shown in both the crosses the silencing of cdk5 causes the down-regulation of Hsp70Aa more evident for the Dauth-Gal4/UAS-Cdk5 line_27517. These results shown clearly that cdk5 regulate Hsp70Aa transcription. Followed the qRT-PCR on RNAi flies stressed for 9h and recovered for 2h to

study the cdk5 and Hsp70Aa gene expression after cold stress (6.17-18).

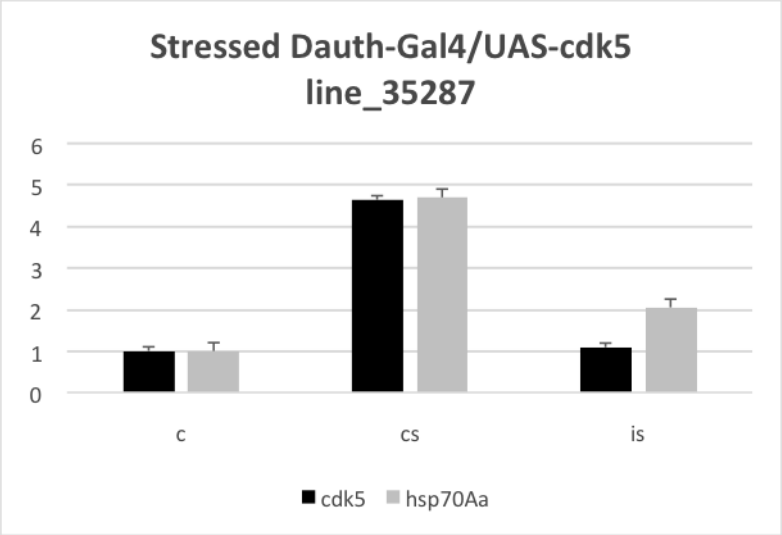

Fig.6.17. Fold change gene expression of Cdk5 and Hsp70Aa in control line and silenced and cold stressed Dauth-Gal4/UAS-Cdk5 line_27517.

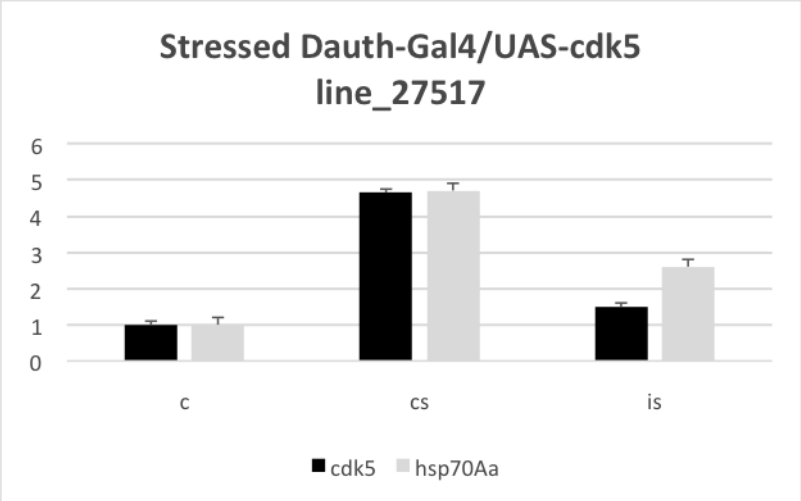

Fig.6.18. Fold change gene expression of Cdk5 and Hsp70Aa in control line and silenced and cold stressed Dauth-Gal4/UAS-Cdk5 line_35287.

As shown in both interfered and stressed lines (fig. 6.17-18) after the 9h of cold stress and 2 h of recovery the cdk5 and Hsp70Aa showed an attenuate expression confirming that the cdk5 gene regulate the Hsp70Aa transcription.

6.5. *Wolbachia* influences on Atg1, Xbp1 and Hsp70aA genes after cold stress

The results reported in the 6.1. section showed that Atg1, Xbp1 in addiction to the Hsp70aA genes were influenced by cold stress. Recently it was reported that the *Wolbachia* infection influenced many cellular pathways of *D. melanogaster* (Zheng et al., 2011). Then, it was studied the possible involvement of *Wolbachia* infection on the manipulation of cellular stress response after cold stress. For this aim, a pool of *Wolbachia*-infected stock (W^+) were treated with antibiotic to obtain a genetically identical *Wolbachia* free *D. melanogaster* line (W^-) (showed in section 5.2). To confirm that *Wolbachia* was removed from treated *D. melanogaster,* it was performed the DNA extractions as described by Bouneb et al., 2014, and PCRs reactions were done using the wsp (table 1, section 5.8) indicated by Braig et al., 1998 for the *Wolbachia* surface protein (wsp) gene.

After the remotion of *Wolbachia* from *D. melanogaster* line (W^-), flies were maintained on standard medium to ensure their full recovery after the antibiotic treatment (Fry et al., 2004). Afterwards W^+ and W^- flies were subjected to cold stress treatment as described by Colinet et al., 2009. The cold stressed flies (W^+ S and W^- S) were then allowed to recover at 25 °C for 2 h. For each treatment, a single *D. melanogaster* female was used for RNA extraction and purification

performed as discussed in section 5.3. Four biological replicates were considered in the analysis. Three hundred nanograms of total RNA were considered for cDNA synthesis performed as previously indicated. Real time-PCRs were carried out using the protocol indicated in section 5.9. The relative quantification of genes expression were evaluated using the $2^{-\Delta\Delta Ct}$ calculation method (Livak and Schmittgen, 2001) (fig. 6.19).

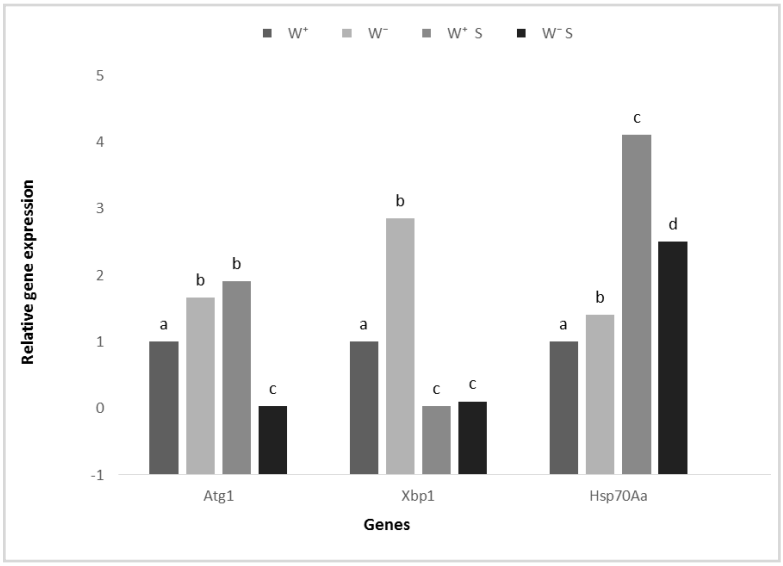

Fig. 6.19. Fold change in gene expression. Different letters between treatments indicate a significant difference in fold change (ANOVA post-hoc test, Tukey; P< 0.05).

The effect of treatment was significant for all three genes (ATG1: $F_{1,\ 3}$ = 27.37, P = 0.000; Xbp1: $F_{1,\ 3}$= 322.79, P= 0.000; Hsp70Aa: $F_{1,\ 3}$ = 277.80, P= 0.000). Prior to cold stress, all the genes were found to be up-regulated in the *Wolbachia*-free *D. melanogaster* line (W^-) in comparison to the *Wolbachia*-infected line (W^+) (Fig.6.19). The difference

between the W^+ and W^- lines could be explained only by the effect of *Wolbachia* and not by the antibiotic treatment (Fry et al., 2004).

The response to cold stress in the *Wolbachia*-infected line was up-regulation of Atg1 (1.90 fold) and Hsp70Aa (4.1 fold) and down-regulation of Xbp1 (0.03 fold). In the *Wolbachia*-free line, we observed a lesser extent of up-regulation of Hsp70Aa (2.5 fold) and down-regulation of Atg1 (0.03 fold) after cold stress. The Xbp1 expression was similar in the W^+ and W^- stressed lines.

6.6. Cold stress influence on the *Wolbachia* wsp gene

The influence of cold stress treatment on *Wolbachia* wsp gene expression has not yet been reported so, it was evaluated the transcriptional changes of the *Wolbachia* wsp gene by means of RT-PCR after 30 min, 2, 4, 6 and 8 h of recovery at 25 °C following a cold stress treatment of 9 h at 0 °C. For this purpose, RNA extraction and cDNA synthesis were carried out as described above and RT-PCR was performed to investigate the expression of the *Wolbachia* wsp gene in infected flies after cold stress. PCR products were loaded on 2% agarose gel and visualized under UV light (fig.6.20).

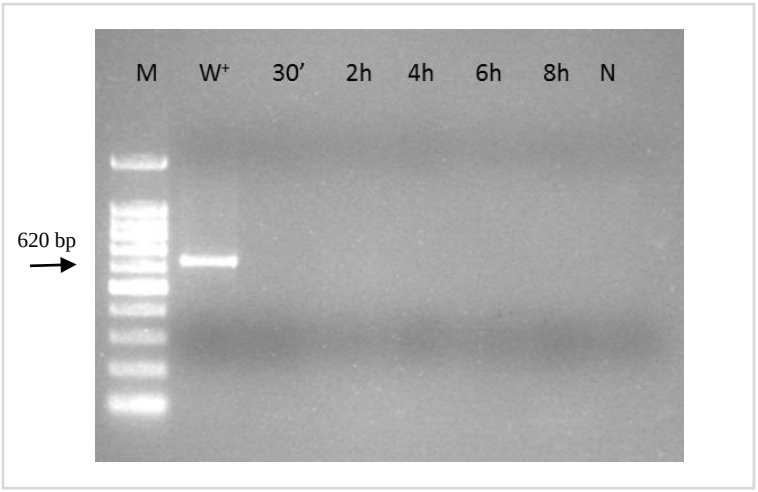

Fig. 6.20. Gel electrophoresis showing the PCR product of the *Wolbachia* wsp gene. Lane 1 (M): 5 µl of marker (100-1500 bp, 5 PRIME); lane 2 (W^+): 10 µl of wsp PCR product (620 bp) of a W^+ fly; lane 3 (30'): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 30 min at 25 °C; lane 4 (2h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 2 h at 25 °C; lane 5 (4h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 4 h at 25 °C; lane 6 (6h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 6 h at 25 °C; lane 7 (8h): 10 µl of PCR product of a W^+ fly stressed for 9 h at 0 °C and allowed to recover for 8 h at 25 °C; lane 8 (N): 10 µl of negative control.

As showed by the fig. 6.20, this treatment negatively influenced the expression of the wsp gene throughout the time course. The wsp gene mRNA could not be detected for up to 8 hours of recovery after cold stress, indicating that *Wolbachia* is very sensitive to cold stress and that its activity is hampered even after recovery of the host.

7. Conclusions

This thesis work starts searching the coding sequences of the key genes involved in the in cellular stress response (CSR): the X box binding protein-1 (Xbp1) for the unfolded protein response by endoplasmic reticulum; the Autophagy-related-gene-1 (Atg1) for the autophagy; the Heat-shock-protein-70Aa (Hsp70Aa) for the heat shock response. qRT-PCR were carried out to verify the involvement of the Atg1 and Xbp1 in addiction to Hsp70Aa in the activation of the CSR after cold stress. Atg1 and Hsp70Aa resulted to be up-regulated by cold stress while Xbp1 down-regulated. After these observations it was decided to focus the attention on the heat shock response and its production of the heat shock proteins among which the Hsp70 family have a key role in the coordination of the CSR. Hence, it was searched a link between the CSR and the cell cycle checkpoints, in which many cyclin/cdk complex are involved. For this purpose it was investigated on the involvement of a new cyclin/cdk complex in the activation of stress response after cold stress. For this reason it was investigated on the Cyclin G (CycG) and the cyclin-dependent kinase 5 (cdk5) as two candidate genes in the activation of heat shock response after the cold stress. For this purpose the timing genes expression of CycG, cdk5 in addition to Hsp70Aa and the Heat shock factor (Hsf) were done after different times of stress and recovery. All the genes studied were influenced by cold stress, generally showing a lower expression in the cold phases in comparison to recovery phases. Afterward,

the timing proteins expression of CycG and the cdk5 was done after different recovery times from stress. Hence, through the co-immunoprecipitation was investigated on the interaction between the CycG and the cdk5 on protein extracts. It was demonstrated that the CycG and cdk5 interacts just in physiological conditions but after the cold stress this interaction became more evident. Moreover it was investigated on the involvement of cdk5 in the transcription regulation of the Heat-shock-protein-70Aa gene by silencing of cdk5 through the RNA interference (RNAi) method using the GAL4/UAS system. After the cross, in the F_1 generation was verified the knock-down of cdk5 gene expression and the basal level of the Hsp70Aa, through the qRT-PCR. The cdk5 resulted silenced with success and the Hsp70Aa gene expression resulted lower than the control line. Then, the RNAi lines were cold stressed and was monitored the cdk5 and Hsp70Aa genes expression after 2 hours of recovery after stress. qRT-PCR showed a cdk5 and Hsp70Aa lower genes expression in RNAi lines cold stressed in comparison to RNAi lines, not cold stressed. These results confirmed that the cdk5 regulates the Hsp70Aa gene expression.

Then, it was studied the possible involvement of *Wolbachia* infection on the manipulation of cellular stress response (CSR) focusing on the unfolded protein response by endoplasmic reticulum, the autophagy and the heat shock response. With this aims, a pool of *Wolbachia*-infected stock (W^+) were treated with

antibiotic, to obtain a genetically identical *Wolbachia* free *D. melanogaster* line (W^-). Through PCR was verified that W^- line was *Wolbachia* free and after were evaluated the Xbp1, the Atg1 and the Hsp70Aa gene expression in W^+ and W^- lines after cold stress and two hours of recovery. All the genes expression resulted to be affected by *Wolbachia* infection influencing the time of recovery after cold stress. It is possible to conclude that *Wolbachia* manipulates the CSR. At last it was investigated on the influence of cold stress treatment on *Wolbachia* surface protein (wsp) gene expression evaluating the transcriptional changes of the *Wolbachia* wsp gene by means of RT-PCR after 30 min, 2, 4, 6 and 8 h of recovery after cold stress. It was emerged that the cold stress treatment negatively influenced the expression of the wsp gene throughout the time course. The wsp gene mRNA could not be detected for up to 8 hours of recovery after cold stress, indicating that *Wolbachia* is very sensitive to cold stress and that its activity is hampered even after recovery of the host.

Concluding, the CSR is more complex and involves many cellular pathways and proteins cross-talker. In future could be interesting the identification of other linker proteins that coordinate and regulate the CSR. Further studies are needed to investigate the molecular mechanism behind the *Wolbachia*-host interaction, the possible involvement of *Wolbachia* in the manipulation of other cellular response pathways and the

mechanism underlying the regulation of *Wolbachia* wsp gene expression after cold stress.

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9. References

Akerfelt M, Morimoto RI, Sistonen L. 2010. Heat shock factors: integrators of cell stress, development and lifespan. Nature reviews Molecular cell biology 11(8):545-555.

Baker BM, Haynes CM. 2011. Mitochondrial protein quality control during biogenesis and aging. Trends in biochemical sciences 36(5):254-261.

Bennin DA, Don AS, Brake T, McKenzie JL, Rosenbaum H, Ortiz L, DePaoli-Roach AA, Horne MC. 2002. Cyclin G2 associates with protein phosphatase 2A catalytic and regulatory B' subunits in active complexes and induces nuclear aberrations and a G1/S phase cell cycle arrest. The Journal of biological chemistry 277(30):27449-27467.

Bouneb M, de Lillo E, Roversi PF, Simoni S. 2014. Molecular detection assay of the bud mite Trisetacus juniperinus on Cupressus sempervirens in nurseries of central Italy. Experimental & applied acarology 62(2):161-170.

Broadley SA, Hartl FU. 2008. Mitochondrial stress signaling: a pathway unfolds. Trends in cell biology 18(1):1-4.

Bukau B, Weissman J, Horwich A. 2006. Molecular chaperones and protein quality control. Cell 125(3):443-451.

Butterworth FM, Emerson L, Rasch EM. 1988. Maturation and degeneration of the fat body in the *Drosophila* larva and pupa as revealed by morphometric analyses. 20: 255-268

Casiraghi M, Bordenstein SR, Baldo L, Lo N, Beninati T, Wernegreen JJ, Werren JH, Bandi C. 2005. Phylogeny of

Wolbachia pipientis based on gltA, groEL and ftsZ gene sequences: clustering of arthropod and nematode symbionts in the F supergroup, and evidence for further diversity in the *Wolbachia* tree. Microbiology 151(Pt 12):4015-4022.

Cecconi F, Levine B. 2008. The role of autophagy in mammalian development: cell makeover rather than cell death. Developmental cell 15(3):344-357.

Cernilogar FM, Onorati MC, Kothe GO, Burroughs AM, Parsi KM, Breiling A, Lo Sardo F, Saxena A, Miyoshi K, Siomi H and others. 2011. Chromatin-associated RNA interference components contribute to transcriptional regulation in *Drosophila*. Nature 480(7377):391-395.

Chirgwin SR, Nowling JM, Coleman SU, Klei TR. 2003. Brugia pahangi and *Wolbachia*: the kinetics of bacteria elimination, worm viability, and host responses following tetracycline treatment. Experimental Parasitology 103(1-2):16-26.

Colinet H, Lee SF, Hoffmann A. 2010. Temporal expression of heat shock genes during cold stress and recovery from chill coma in adult *Drosophila melanogaster*. The FEBS journal 277(1):174-185.

Cooke MS, Evans MD, Dizdaroglu M, Lunec J. 2003. Oxidative DNA damage: mechanisms, mutation, and disease. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 17(10):1195-1214.

Craig EA.1988. The heat shock response. CRC Critical Review in Biochemistry 18 (3): 239-80.

Dérijard B, Hibi M, Wu IH, Barret T, Su B, Deng T, Karin M, Davis RJ. 1994. Cell 76 (6): 1025-1037.

Deretic V. 2010. Autophagy of intracellular microbes and mitochondria: two sides of the same coin? F1000 biology reports 2.

Duffy JB. 2002. GAL4 system in Drosophila: a fly geneticist's Swiss army knife. Genesis 34(1-2):1-15.

Duncan RF. 2005. Inhibition of Hsp90 function delays and impairs recovery from heat shock. The FEBS journal 272(20):5244-5256.

Eleftherianos I, Atri J, Accetta J, Castillo JC. 2013. Endosymbiotic bacteria in insects: guardians of the immune system? Frontiers in physiology 4:46.

Faradji F, Bloyer S, Dardalhon-Cuménal D, Randsholt NB, Peronnet F. 2014. *Drosophila melanogaster*Cyclin G coordinates cell growth and cell proliferation. Cell Cycle 10(5):805-818.

Fry AJ, Palmer MR, Rand DM. 2004. Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. Heredity (Edinb) 93(4):379-389.

Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, Li Y, Hao YL, Ooi CE, Godwin B, Vitols E and others. 2003. A protein interaction map of *Drosophila melanogaster*. Science 302(5651):1727-1736.

Girardot F, Monnier V, Tricoire H. 2004. Genome wide analysis of common and specific stress responses in adult *Drosophila melanogaster*. BMC Genomics 5:74.

Halliwell B, Gutterdidge GJ. 1996. Free Radicals in Biology and Medicine. Clarendon press. Oxford.

Hance T, van Baaren J, Vernon P, Boivin G. 2007. Impact of extreme temperatures on parasitoids in a climate change perspective. Annual review of entomology 52:107-126.

Hang H, He L, Fox MH. 1995. Cell cycle variation of Hsp70 levels in HeLa cells at 37 °C and after a heat shock. Journal of Cellular Physiology 165:367–75.

Hara T, Nakamura K, Matsui M, Yamamoto A, Nakahara Y, Suzuki-Migishima R, Yokoyama M, Mishima K, Saito I, Okano H and others. 2006. Suppression of basal autophagy in neural cells causes neurodegenerative disease in mice. Nature 441(7095):885-889.

Haynes CM, Yang Y, Blais SP, Neubert TA, Ron D. 2010. The matrix peptide exporter HAF-1 signals a mitochondrial UPR by activating the transcription factor ZC376.7 in C. elegans. Molecular cell 37(4):529-540.

Hoeijmakers JH. 2009. DNA damage, aging, and cancer. New England Journal of Medicine 361 (19): 1914.

Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, Muzzi F, Greenfield M, Durkan M, Leong YS, Dong Y and others. 2011. Successful establishment of

Wolbachia in Aedes populations to suppress dengue transmission. Nature 476(7361):454-457.

Hoffman and Parson. 1991. Evolutionary Genetics and Environmental Stress. Oxford University Press.

Hoffmann AA, Sørensen JG, Loeschcke V. 2003. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. Journal of Thermal Biology 28(3):175-216.

Høyer-Hansen M, Jäättelä M. 2014. Autophagy: An emerging target for cancer therapy. Autophagy 4(5):574-580.

Hung RJ, Hall J, Brennan P, Boffetta P. 2005. Genetic polymorphisms in the base excision repair pathway and cancer risk: a HuGE review. American journal of epidemiology 162(10):925-942

Hunt T. 1991. Cyclins and their partners from a simple idea to complicated reality. Seminars in Cell Biology 2(4): 213-222.

Kiang JG, Tsokos C. 1996. Cell Signaling and Heat Shock Protein Expression. Journal of Biomedical Science 3:379-388.

Jerome V, Vourc'h C, Baulieu EE, Catelli MG. 1993. Cell cycle regulation of the chicken hsp90 alpha expression. Experimental Cell Research 205:44–51.

Jung CH, Ro SH, Cao J, Otto NM, Kim DH. 2010. mTOR regulation of autophagy. FEBS letters 584(7):1287-1295.

Komatsu M, Waguri S, Chiba T, Murata S, Iwata J, Tanida I, Ueno T, Koike M, Uchiyama Y, Kominami E and others. 2006.

Loss of autophagy in the central nervous system causes neurodegeneration in mice. Nature 441(7095):880-884.

Kostal V, Renault D, Mehrabianova A, Bastl J. 2007. Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of ion homeostasis. Comparative biochemistry and physiology Part A, Molecular & integrative physiology 147(1):231-238.

Kourtis N, Tavernarakis N. 2011. Cellular stress response pathways and ageing: intricate molecular relationships. The EMBO journal 30(13):2520-2531.

Kroemer G, Marino G, Levine B. 2010. Autophagy and the integrated stress response. Molecular cell 40(2):280-293.

Krokan HE, Nilsen H, Skorpen F, Otterlei M, Slupphaug G. 2000. Base excision repair of DNA in mammalian cells. FEBS Letters 476: 73-77.

Kultz D. 2003. Evolution of the cellular stress proteome: from monophyletic origin to ubiquitous function. Journal of Experimental Biology 206(18):3119-3124.

Kultz D. 2005. Molecular and evolutionary basis of the cellular stress response. Annual review of physiology 67:225-257.

Kultz D, Madhany S, Burg MB. 1998. Hyperosmolality Causes Growth Arrest of Murine Kidney Cells: induction of gadd45 and gadd153 by osmosensing via stress-activated protein kinase 2. Journal of Biological Chemistry 273(22):13645-13651.

Lai E, Teodoro T, Volchuk A. 2007. Endoplasmic reticulum stress: signaling the unfolded protein response. Physiology 22:193-201.

Lee C-Y, Cooksey BAK, Baehrecke EH. 2002. Steroid Regulation of Midgut Cell Death during Drosophila Development. Developmental Biology 250(1):101-111.

Levine B. 2005. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. Cell 120(2):159-162.

Levine B, Kroemer G. 2008. Autophagy in the pathogenesis of disease. Cell 132(1):27-42.

Levine B, Yuan J. 2005. Autophagy in cell death: an innocent convict? The Journal of clinical investigation 115(10):2679-2688.

Li F, Vierstra RD. 2012. Autophagy: a multifaceted intracellular system for bulk and selective recycling. Trends in plant science 17(9):526-537.

Lindquist S and Craig EA. 1988. The heat-shock proteins. Annual Review of Genetics 22:631-677.

Livak KJ, Schmittgen TD. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25(4):402-408.

Lum JJ, DeBerardinis RJ, Thompson CB. 2005. Autophagy in metazoans: cell survival in the land of plenty. Nature reviews Molecular cell biology 6(6):439-448.

Mammucari C, Milan G, Romanello V, Masiero E, Rudolf R, Del Piccolo P, Burden SJ, Di Lisi R, Sandri C, Zhao J and others. 2007. FoxO3 controls autophagy in skeletal muscle in vivo. Cell metabolism 6(6):458-471.

Mathew R, Kongara S, Beaudoin B, Karp CM, Bray K, Degenhardt K, Chen G, Jin S, White E. 2007. Autophagy suppresses tumor progression by limiting chromosomal instability. Genes & development 21(11):1367-1381.

Melendez A, Neufeld TP. 2008. The cell biology of autophagy in metazoans: a developing story. Development 135(14):2347-2360.

Mizushima N, Levine B, Cuervo AM, Klionsky DJ. 2008. Autophagy fights disease through cellular self-digestion. Nature 451(7182):1069-1075.

Morimoto RI. 2008. Proteotoxic stress and inducible chaperone networks in neurodegenerative disease and aging. Genes & development 22(11):1427-1438.

Neal SJ, Karunanithi S, Best A, So AK, Tanguay RM, Atwood HL, Westwood JT. 2006. Thermoprotection of synaptic transmission in a Drosophila heat shock factor mutant is accompanied by increased expression of Hsp83 and DnaJ-1. Physiological genomics 25(3):493-501.

Nilson TL, Sinclair BJ, Roberts SP. 2006. The effects of carbon dioxide anesthesia and anoxia on rapid cold-hardening and

chill coma recovery in *Drosophila melanogaster*. J Insect Physiol 52(10):1027-1033.

Pandey UB, Nichols CD. 2011. Human disease models in *Drosophila melanogaster* and the role of the fly in therapeutic drug discovery. Pharmacological reviews 63(2):411-436.

Parsell and Lindquist. 1993. The function of the Heat-Shock Proteins in stress tolerance: Degradation and Reactivation of Damaged Proteins. Annual Review of Genetics 27:437-496.

Pellegrino MW, Nargund AM, Haynes CM. 2013. Signaling the mitochondrial unfolded protein response. Biochimica et biophysica acta 1833(2):410-416.

Pines J and Hunter T. 1989. Isolation of a human cyclin cDNA: Evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cc2. Cell 58 (5):833-846.

Poinsot D, Charlat S, Mercot H. 2003. On the mechanism of Wolbachia-induced cytoplasmic incompatibility: confronting the models with the facts. Bioessays 25(3):259-265.

Postel-Vinay S, Vanhecke E, Olaussen KA, Lord CJ, Ashworth A, Soria JC. 2012. The potential of exploiting DNA-repair defects for optimizing lung cancer treatment. Nature reviews Clinical oncology 9(3):144-155.

Rasheva VI, Domingos PM. 2009. Cellular responses to endoplasmic reticulum stress and apoptosis. Apoptosis : an international journal on programmed cell death 14(8):996-1007.

Rinehart JP, Li A, Yocum GD, Robich RM, Hayward SA, Denlinger DL. 2007. Up-regulation of heat shock proteins is essential for cold survival during insect diapause. Proceedings of the National Academy of Sciences of the United States of America 104(27):11130-11137.

Ron D, Walter P. 2007. Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews Molecular cell biology 8(7):519-529.

Rubinsztein DC, Gestwicki JE, Murphy LO, Klionsky DJ. 2007. Potential therapeutic applications of autophagy. Nature reviews Drug discovery 6(4):304-312.

Salvaing J, Nagel AC, Mouchel-Vielh E, Bloyer S, Maier D, Preiss A, Peronnet F. 2008. The enhancer of trithorax and polycomb corto interacts with cyclin G in Drosophila. PLoS One 3(2):e1658.

Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. 2004. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annual review of biochemistry 73:39-85.

Scott RC, Juhasz G, Neufeld TP. 2007. Direct induction of autophagy by Atg1 inhibits cell growth and induces apoptotic cell death. Current biology : CB 17(1):1-11.

Seno JD, Dynlacht JR. 2004. Intracellular redistribution and modification of proteins of the Mre11/Rad50/Nbs1 DNA

repair complex following irradiation and heat-shock. Journal of cellular physiology 199(2):157-170.

Simmons SO, Fan CY, Ramabhadran R. 2009. Cellular stress response pathway system as a sentinel ensemble in toxicological screening. Toxicological sciences : an official journal of the Society of Toxicology 111(2):202-225.

Sinclair BJ, Vernon P, Jaco Klok C, Chown SL. 2003. Insects at low temperatures: an ecological perspective. Trends in Ecology & Evolution 18(5):257-262.

Sørensen JG, Kristensen TN, Loeschcke V. 2003. The evolutionary and ecological role of heat shock proteins. Ecology Letters 6(11):1025-1037.

SPSS, 2004. SPSS Guide, v. 13.0. Chicago, Ill.

Starokadomskyy P, Dmytruk KV. 2013. A bird's-eye view of autophagy. Autophagy 9(7):1121-1126.

Tamura K, Kanaoka Y, Jinno S, Nagata A, Ogiso Y, Shimizu K, Hayakawa T, Nojima H, Okayama H. 1993. Cyclin G a new mammalian cyclin with homology to fissation yest Cig1. Oncogene 8 (8): 2113-8.

Thorpe GW, Fong CS, Alic N, Higgins VJ, Dawes IW. 2004. Cells have distinct mechanisms to maintain protection against different reactive oxygen species: oxidative-stress-response genes. Proceedings of the National Academy of Sciences of the United States of America 101(17):6564-6569.

Turturici G, Sconzo G, Geraci F. 2011. Hsp70 and its molecular role in nervous system diseases. Biochemistry research international 2011:618127.

van der Putten WH, de Ruiter PC, Martijn Bezemer T, Harvey JA, Wassen M, Wolters V. 2004. Trophic interactions in a changing world. Basic and Applied Ecology 5(6):487-494.

Walker T, Johnson PH, Moreira LA, Iturbe-Ormaetxe I, Frentiu FD, McMeniman CJ, Leong YS, Dong Y, Axford J, Kriesner P and others. 2011. The wMel *Wolbachia* strain blocks dengue and invades caged Aedes aegypti populations. Nature 476(7361):450-453.

Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat Rev Microbiol 6(10):741-751.

Wu C, Clos J, Giorgi G, Haroun RI, Kim SJ, Rabindran SK, Westwood T, Wisniewski J, and Yim G. 1994. The Biology of Heat Shock Proteins and Molecular Chaperones. Cold Spring Harbor 26.

Wu H, Wang MC, Bohmann D. 2009. JNK protects *Drosophila* from oxidative stress by trancriptionally activating autophagy. Mech Dev 126(8-9):624-637.

Xiong Y, Contento AL, Nguyen PQ, Bassham DC. 2007. Degradation of oxidized proteins by autophagy during oxidative stress in Arabidopsis. Plant physiology 143(1):291-299.

Yorimitsu T, Klionsky DJ. 2005. Autophagy: molecular machinery for self-eating. Cell death and differentiation 12 Suppl 2:1542-1552.

Yorimitsu T, Nair U, Yang Z, Klionsky DJ. 2006. Endoplasmic reticulum stress triggers autophagy. The Journal of biological chemistry 281(40):30299-30304.

Young JC, Agashe VR, Siegers K, Hartl FU. 2004. Pathways of chaperone-mediated protein folding in the cytosol. Nature reviews Molecular cell biology 5(10):781-791.

Yue Z, Jin S, Yang C, Levine AJ, Heintz N. 2003. Beclin 1, an autophagy gene essential for early embryonic development, is a haploinsufficient tumor suppressor. Proceedings of the National Academy of Sciences of the United States of America 100(25):15077-15082.

Zamore PD, Tuschl T, Sharp PA, Bartel DP. 2000. RNAi. Cell 101(1):25-33.

Zauberman A, Lupo A, Oren M. 1995. Identification of p53 target genes through immune selection of genomic DNA: the cyclin G gene contains two distinct p53 binding sites. Oncogene 10 (12): 2361-6.

Zhao J, Brault JJ, Schild A, Cao P, Sandri M, Schiaffino S, Lecker SH, Goldberg AL. 2007. FoxO3 coordinately activates protein degradation by the autophagic/lysosomal and proteasomal pathways in atrophying muscle cells. Cell metabolism 6(6):472-483.

Zheng Y, Wang JL, Liu C, Wang CP, Walker T, Wang YF. 2011. Differentially expressed profiles in the larval testes of *Wolbachia* infected and uninfected *Drosophila*. BMC Genomics 12:595.

10. Supplementary materials

10.1. Cell extraction buffer composition (Invitrogen™)

- 10 mM Tris, pH 7.4
- 100 mM NaCl
- 1 mM EDTA
- 1 mM EGTA
- 1 mM NaF
- 20 mM $Na_4P_2O_7$
- 2 mM Na_3VO_4
- 1 % Triton X-100
- 10 % glycerol
- 0.1 % SDS
- 0.5 % deoxycholate
-

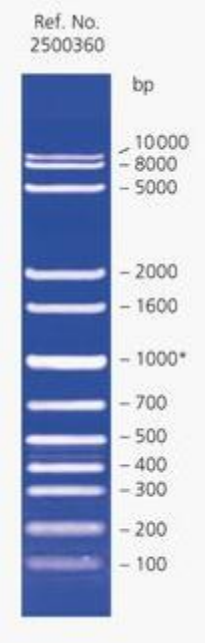
10.2. Sample buffer 2X

- 20X NuPage® LDS Sample Buffer (Life technologies™)
- 10X NuPage® Sample Reducing Agent (Life technologies™)

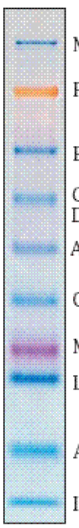
10.3. TEA_{150} buffer

- 10 mM Tris pH 8,
- 150 mM NaCl,
- 1 mM EDTA, 1 mM DTT,
- 1 mM PMSF,
- 0.1 % NP40.

10.4. DNA ladder 100-1500 bp, 5 PRIME



10.5. SeeBlue® Plus2 Pre-Stained Standards (Life technologies™)



Protein	Approximate Molecular Weights (kDa)				
	Tris-Glycine	Tricine	NuPAGE® MES	NuPAGE® MOPS	NuPAGE® Tris-Acetate
Myosin	250	210	188	191	210
Phosphorylase	148	105	98	97	111
BSA	98	78	62	64	71
Glutamic Dehydrogenase	64	55	49	51	55
Alcohol Dehydrogenase	50	45	38	39	41
Carbonic Anhydrase	36	34	28	28	n/a
Myoglobin Red	22	17	17	19	n/a
Lysozyme	16	16	14	14	n/a
Aprotinin	6	7	6	n/a	n/a
Insulin, B Chain	4	4	3	n/a	n/a

NuPAGE® Novex
Bis-Tris 4-12% Gel

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