Age related changes in translational regulation and Endoplasmic Reticulum (ER) stress response

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By

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Dedicated to my dearest parents

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DECLARATION

I hereby declare that the work presented in this thesis entitled **"Age related changes in translational regulation and Endoplasmic Reticulum (ER) stress response"** has been carried out by me under the supervision of Prof. K. V. A. Ramaiah and this work has not been submitted for any degree or diploma of any university.

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CERTIFICATE

This is to certify that Syed Gulam Hussain has carried out the work in the present thesis under my supervision for a full period prescribed under the PhD ordinance of the university. I recommend his thesis entitled **"Age related changes in translational regulation and Endoplasmic Reticulum (ER) stress response"** for submission for the award of the degree of Doctor of Philosophy of this university.

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Abbreviations:

AP : Alkaline phosphate
ATF4 : Activating transcription factor 4
ATP : Adenosine 5' triphosphate
Bad : Bcl-xL/Bcl-2 associated death promoter
Bak : Bcl-2 homologous antagonist/killer
BAP31 : BiP-associated protein 31
Bax : Bcl-2 associated X protein
BCIP : 5-bromo-4-chloro-3-indoyl phosphate
Bcl-2 : B cell leukemia/lymphoma 2
Bcl-xL : Bcl-x long
BH : Bcl-2 homology domain
Bim : Bcl-2 interacting mediator of cell death
BiP : immuglobulin heavy chain binding protein
bZIP : basic leucine zipper
C/EBP : CCAAT-enhancer binding protein
c-Abl : cellular Abelson murine leukemia virus transforming protein
cAMP : cyclic adenosine monophosphate
CAT-1 : cationic amino acid transporter
CHOP : C/EBP-homologous protein
CREB : CRE binding protein
CreP : constitutive repressor of eIF2α phosphorylation
eIF : eukaryotic translation initiation factor
ERAD : ER associated degradation
ERK : extracellular signal regulated kinase
FADD : Fas associated protein with a novel death domain
GADD153 : growth arrest and DNA damage-inducible 153 gene
GADD34 : growth arrest and DNA damage-inducible gene 34
GCN : general control non-derepressible
GDP : guanosine diphosphate
GLS : golgi localization sequence
GRP : glucose regulated protein
GSH : Reduced glutathione

GST : glutathione S-transferase

GTP : guanosine triphosphate

HAC1 : homologous to ATF/CREB1

HRI : Heme regulated inhibitor

IFN : Interferons

IκB : inhibitor of NF-κB

IRE1 : inositol requiring 1

IRES : internal ribosomal entry site

JAB1 : Jun activation domain binding protein-1

JNK : Jun N-terminal kinase

MAPK : mitogen-activated protein kinase

MNK : MAP interacting kinase

mRNA : messenger ribonucleic acid

NBT : Nitro blue tetrazolium

NF-κB : nuclear factor κB

nm : nanometers

NRF2 : NF-E2 related factor

Pi : inorganic phosphate

$P58^{IPK}$: 58 kDa inhibitor of PKR

PABP : polyA binding protein

PDI : protein disulfide isomerase

rpm : rotations per minute

SDS : sodium dodecyl sulphate

Sf9 : *Spdoptera frugiperdra*

TEMED : N'N'N'N'N'-tetra ethyl methyl ethyl diamine

TRAF2 : tumor necrosis factor receptor associated factor 2

tRNA : transfer RNA

uORF : upstream open reading frame

UPR : unfolded protein response

XBP-1 : X box binding protein

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Aging can be interpreted in many different ways; however it is different from disease. Chronological aging is characterized by a general decline in physiological function and by a decline in the ability to maintain homeostatic balance (Sartin et al. 1980). Age associated progressive decline in homeostasis and in the ability to respond to stress increases the susceptibility of an aged organism to a plethora of diseases and eventual death upon insult (Rattan, 2000a; 2000b; 2004). Theories formulated to explain aging processes have been grouped as intrinsic or extrinsic (Weinert and Timiras, 2003) and are linked to evolutionary, genetic, molecular, cellular, environmental and system levels.

1.1: Theories of aging:

Evolutionary theories signify aging as an adaptive measure to overcrowding population. The evolutionary theory was postulated in the 1940s based on the observation that Huntington's disease, a dominant lethal mutation remained in the population even though it should have been strongly selected against. The late onset of the disease allows it to avoid the force of natural selection and pass on the genes to next generation. The mutation accumulation theory is also inspired by the similar premise and suggests that detrimental late acting mutations accumulate in the population and ultimately lead to pathology and death (Weinert and Timiras, 2003). The disposable soma theory argues that somatic organism is maintained only for reproductive success later on it is disposed off. This theory explains, why we live but does not depict why or how we die. In contrast, the antagonistic pleiotropy theory suggests that some genes are selected for beneficial effects early in life and with age have deleterious effects leading to senescence (Weinert and Timiras, 2003).

Molecular theories argue that aging is caused by, 1; genes that regulate development and aging (Gene regulation theory), 2; loss in the fidelity of translation due to inefficient decoding of codons (Codon restriction theory), 3; increased accumulation of abnormal proteins due to decline in the fidelity of gene expression (Error catastrophe theory), 4; molecular damage accumulation due to damage to genetic material (Somatic mutation theory), and 5; impaired gene expression due to accumulation of damaged macromolecules (Dysdifferentiation theory) (Weinert and Timiras, 2003).

The **cellular theories** such as cellular senescence theory, suggest that the phenotype of aging is caused by an increase in the number of terminally arrested senescent cells with altered physiology after characteristic number of cell division (Campisi, 2003).

The replicative senescence or telomere theory suggest that the shortening and altering of telomere length and structure with every cell division leads to cellular senescence (Blackburn, 2000). Specialized immortal cell types such as stem cells, germ cells, T-lymphoctes, etc, express telomerase and either maintain telomere length or delay telomere shortening (Collins, 2000). Stress induced senescence occurs in response to stressors that damage DNA, modify heterochromatin, and induce strong mitogen signaling (Campisi, 2003). However there is ongoing debate on the relative contributions of replicative senescence and stress induced senescence in the aging process. The cellular senescence theory may complement with the various damage accumulation theories like free radical, error catastrophe, and somatic mutation theories, ultimately leading to senescence during aging.

Free radical theory was first proposed in 1957 and is one of most common theory to date (Harman, 1957). All organisms live in an environment rich in free radicals containing reactive oxygen species (ROS). Cellular processes like mitochondrial respiration and endoplasmic reticulum (ER)-associated protein folding also generate ROS (Finkel and Hoolbrook, 2000; Tu and Weissman, 2004). Sustained damage inflicted by endogenously produced oxidants/free radicals during various metabolic processes epigenetically contribute to aging and its related deficits (Shigenaga et al. 1994). The free radicals damage cellular macromolecules, including DNA (Fraga et al. 1990), protein (Stadtman, 1992), and lipids (Marnett, 1985). The oxidative free radicals are generally scavenged by super oxide dismutase 1 (SOD 1) (Finkel and Holbrook, 2000), in addition, the anti-oxidant defence mechanisms possessed by cell maintain redox homeostasis (Droge, 2002). However, this defence approach is far from efficient in completely eliminating the oxidants during severe insults and its efficiency is shown to reduce with age (Finkel and Holbrook, 2000, Miura, 2004). Free radical scavenging enzymes are sufficient to delay aging in *Drosophila* (Tower, 2000) and the flies that have enhanced expression of SOD1 and high resistance to oxidative stress have elongated lifespan (Arking et al. 2000). Calorie restriction or reduced dietary intake reduces metabolism related ROS generation promoting longevity. Although it is easy to speculate a correlation between physiologic function and calorie restriction, it is difficult to point out the ultimate cause for longevity due to several molecular and cellular changes that accompany it. The free radical theory is further categorized on the basis of the particular organelle and type of macromolecule damaged during the aging process.

The **wear and tear theory** emphasizes the importance of maintenance systems in an organism to prevent the accumulation of injury over a period of time. The better the maintenance, the more the organism approaches immortality. The a**poptosis theory** however links programmed cell death triggered by genetic events to be associated with aging and death (Weinert and Timiras, 2003).

System-based theories of aging relate decline in the function of organ systems to be associated with a consecutive decline in the maintenance of other dependent systems to be responsible for aging. Neuroendocrine theory proposes that aging is due to changes in neural endocrine functions that are crucial for i) communication and responsiveness of all body systems with the external environment, ii) physiological responses and maintenance of optimal functional state responding to environmental demands. These changes not only affect the neurons and hormones that regulate reproduction, growth and development but also affect those systems that regulate survival through adaptation to stress. The life span functions as cyclic body regulated by, "biological clocks". Alterations that disrupt the clock, such as decreased responsive to stimuli that drive the clock and excessive or insufficient coordination of responses leads to aging. The hypothalamo-pituitary-adrenal (HPA) axis functions as a master regulator mustering physiological adjustments necessary for maintenance of internal homeostasis against environmental disturbances (Weinert and Timiras, 2003). Chronic exposure to different forms of severe stress during life span from a mulititude of physical, biological or emotional stimuli may exhaust and weaken the capacity to adapt and lead to aging (Selye, 1976). The neuroedocrine theory is supported by experiments in *C. elegans* that have shown insulin pathway to play a role in stress response and longevity (Kawano et al. 2000). The neuroendocrine and immune systems interact significantly and in tandem to the neuroendocrine system, the immune system serves several essential functions. The immune system controls and eliminates foreign organisms and substances in host body and at the same time recognizes and spares self molecules. Different immune responses are differentially affected with age. The thymus, that is involved in T-cell selection and maturation reaches a peak during puberty and atrophies thereafter (George and Ritter, 1996). Elderly humans show characteristics of immunosenescence such as decreased resistance to infectious diseases and an increased failure to recognize self molecules (Franceschi et al. 2000), suggesting a role of decline in the function of immune system in aging (Weinert and Timiras, 2003).

From the above content, it is quite obvious that the ultimate cause of aging is still unknown and may require integration of various models and theories to account for normal aging. In complex multicellular organisms, theories that abridge the interaction among intrinsic (genetic), extrinsic (environmental) and stochastic (random damage to vital molecules) causes comprehensively describe the aging process. In recent years, the search for a single cause of aging, such as a gene or a key body system, has been replaced by the view that aging is a multi-factorial phenomenon that involves simultaneous interaction of many complex processes operating at various levels of functional organization (Franceschi et al. 2000). Hence a unified theory of biological aging that sums up the failure of homeodynamics, genes, and environmental influence, is widely gaining acceptance (Rattan, 2006).

The rate of aging is highly variable, among different species or within a species, in organs within an organism, in cell types within a tissue, in sub-cellular compartments within a cell, and among macromolecules within a cell (Rattan, 2000a; 2000b). Gross mechanisms of aging can certainly differ in different organisms, specifically at molecular level. For example, accumulation of extrachromosomal ribosomal DNA circles and extrachromosomal mtichondrial DNA circles contributes to aging in yeast and filamentous fungi respectively (Sinclair and Guarente, 1997; Osiewacsz, 2002). However, neither of these mechanisms contribute to aging in mammals.

1.2: Aging and Protein synthesis.

Decline in bulk protein synthesis is one of the most common biochemical change observed during aging and there is a considerable variability in the extent of decline, varying from 20% to 80% among different tissues and cell types (Garcia et al. 1994; Ward and Richardson, 1991; Rattan, 1992). The implications and consequences of this detrimental change in protein synthesis are manifold, and include a decline in the availability of enzymes for the maintenance, repair, and normal metabolic functions, inefficient removal of abnormal, and damaged macromolecules, inefficient intracellular and intercellular signaling, reduced production and secretion of hormones, antibodies, neurotransmitters, and a decline in proliferative capacity (Rattan, 1996). It is important to note that while general, or total, protein synthesis does decrease with age, the protein synthetic rates in different tissues as well as the synthesis of specific individual proteins may decrease, not change or may actually increase. For example, protein synthesis in soleus muscle of Fischer rat has been found to decrease with age while, in the same animal, the synthetic rate in the gastrocnemius and extensor

digitorus longus muscles was unchanged (Fluckey et al. 1996). Age-related changes in protein synthesis can be achieved by regulation at the transcriptional and pretranslational levels in terms of the availability of individual mRNA species for translation, and at the translational and posttranslational levels in terms of alterations in the components of the protein synthetic machinery.

1.3: Overview of Translation:

Translation is the process of generating proteins by polymerizing amino acids according to the instructions read from messenger RNA (mRNA). Translation is accomplished by moving the mRNA through a groove in the ribosome in concert with a considerable number of accessory factors. The ribosome is a large ribonucleoprotein complex, composed of ribosomal RNA's and 78 different proteins. It is organized in two subunits: 40S and 60S subunits. The amino acids are brought by corresponding tRNAs on to the mRNA template bound by ribosomes and translational factors, one after another where they are finally polymerized. Amino-acyl synthetase enzymes charge the corresponding tRNAs with amino acids, and the charged tRNAs specifically deliver the amino acids by recognizing the cognate codon on mRNA template through its anticodon. The dynamic process of mRNA translation is usually divided into four stages: initiation, elongation, termination and recycling (Kapp and Lorsch, 2004).

1.3.1: Initiation: The initiation phase represents all the processes required for the assembly of an initiator-methionyl-transfer-RNA (Met-tRNAi^{Met}) in the peptidyl (P-) site of ribosome and on the start codon of the mRNA. Translation initiation is accomplished in four successive steps: (i) formation of a heterotrimeric 43S (43 Svedberg) preinitiation complex from the small (40S) ribosomal subunit, initiation factors, and Met-tRNAi^{Met}, (ii) recruitment of the 43S complex to the 5' end of the capped mRNA, (iii) 'scanning' of the 5' untranslated region (UTR) of the mRNA and start codon recognition, and (iv) joining of a large (60S) subunit to assemble a complete (80S) ribosome. At least 12 eukaryotic initiation factors (eIF's) play a role in eukaryotic translation initiation (Kyrpides and Woese, 1998).

Formation of 43S preinitiation complex: eIF2 is a heterotimer, made up of three different subunits α, β and γ, with a total mol wt of ~125 kDa. Ternary complex formation is accomplished when eIF2 in its GTP bound form binds to initiator Met-tRNAi. Biochemical and genetic studies suggest that the γ subunit of eIF2 is the site

for the binding of both GTP and Met-tRNAi (Kapp and Lorsch, 2004). The β-subunit promotes GTPase activity and modulates initiator tRNA binding of eIF2γ. The eIF2β subunit is also implicated in mRNA binding and in codon–anticodon interactions and in exclusive interaction with the eIF2 interacting partners eIF5 and eIF2B (Laurino et al. 1999; Asano et al. 1999). The α-subunit of eIF2 functions predominantly as a regulator of translation initiation and is required for modulating the interaction between eIF2 and its guanine nucleotide exchange factor eIF2B. Recently it has been shown that eIF2 in its GTP-bound form has a positive contact with the methionine on the initiator tRNA and that this contact is disrupted when GTP is exchanged for GDP (e.g., upon GTP hydrolysis), whereas contacts to the body of the tRNA are not altered (Kapp and Lorsch, 2004). This GTP-dependent recognition of the methionine moiety may in part prevent unacylated tRNAi from entering the initiation pathway and is also likely to play role in release of the initiator tRNA from eIF2 upon initiation codon recognition. eIF1, eIF1A and eIF3 assist in the binding of eIF2-GTP-Met-tRNAi ternary complex to the 40S ribosomal subunit.

eIF3, the largest of the initiation factors, is a multimeric protein of about 600 kDa and constituted of 11 subunits in mammals (Browning et al. 2001). eIF3 is a hub of interactions and interacts with eIFs 1, 1A, 2, 4B, 4G, and 5 as well as with the 40S subunit. These interactions suggest that eIF3 functions as a central organizer of the translation initiation machinery coordinating complicated arrangements of components required to put both the ternary complex and mRNA on the ribosome, while preventing the large ribosomal subunit from prematurely joining the small ribosomal subunit. A variety of experiments have shown that eIFs 1, 2, 3, 5, and Met-tRNAi (presumably bound to eIF2) exists stably *in-vivo* as a "multifactor complex" (MFC) (Asano et al. 2000) and that the integrity of this complex is important for translation initiation (Kapp and Lorsch 2004). eIF1A is a small highly polar protein of 17 to 22 kDa (Wei et al., 1995). It binds RNA in a random manner, either to mRNA or rRNA and is implicated in promoting ribosome dissociation, ternary complex recruitment to the 40S subunit and in mRNA binding and scanning (Pestova et al. 1998; Chaudhuri et al. 1999). eIF5 is a GTPase activating protein (GAP) that stimulates the intrinsic GTPase activity of eIF2 complex. It promotes ternary complex recruitment and/or mRNA binding to the 40S subunit. eIF5 is a central component of the MFC, interacting with the ternary complex in addition to eIFs 1, 3 and 2 (Kapp

and Lorsch 2004). Apart from stabilizing the 43S preinitiation complex, these factors may also facilitate the 43S complex formation by altering the conformation of the ribosome either locally or globally. Small factors such as eIFs 1 and 1A might alter the local conformation of the eIF2 binding site, thought to be over and around the P site (Bommer et al. 1988), whereas eIF3 might distort the conformation of the entire 40S subunit to allow easier access of eIF2 with its attached Met-tRNAi (Kapp and Lorsch 2004).

Recruitment of the 43S complex to the 5' end of the mRNA: Unlike prokaryotes, eukaryotic translation is uncoupled temporally and spatially from transcription. The mRNA transcript is synthesized in the nucleus and then processed by capping, splicing and polyadenylation before being exported as a messenger ribonucleoprotein (mRNP) complex into the cytoplasm through nuclear pores. mRNA in mRNP particles is highly structured ruling out the possibility of a mechanism similar to prokaryotes for recognition of initiation site. Unlike the prokaryotes that have the Shine-Dalgarno initiation codon identification system, eukaryotes have evolved a mechanism referred as scanning model for recognition of initiation site (Kozak and Shatkin, 1978). The loading of the mRNA onto the 43S complex is performed by eIF4F complex constituted primarily of eIF4E, eIF4G and eIF4A (Grifo et al. 1983), in conjunction with eIF3 and the poly(A) binding protein (PABP) bound to the 3'-poly(A) tail. The eIF4F complex performs a two fold role, initially it recruits eIF4A to unwind secondary structures in the 5'-end of the mRNA and make a suitable binding site for the 43S complex. Later it brings factors such as eIF3 to the 5'-end of the mRNA that facilitates the binding of 43S complex to the newly ironed out mRNA (Lamphear et al. 1995). The first step in loading of the mRNA to the 43S complex is the recognition of the 7-methylguanosine cap on the 5' end of mRNA by the cap binding protein eIF4E. The eIF4E resembles a cupped hand and its concave side provides a small hydrophobic slot for insertion of the cap structure and a contiguous region for mRNA binding where as the convex face of eIF4E interacts with eIF4G (Marcotrigiano et al. 1997; Matsuo et al. 1997). The centrepiece of eIF4F is the multivalent adapter molecule eIF4G (Hentze, 1997). eIF4G functions similar to eIF3 as a hub of interaction facilitating the assembly of eIF4F complex. The N-terminal region of eIF4G harbours binding sites for eIF4E and PABP (Sachs and Varani, 2000). The central region of eIF4G interacts with eIF4A, RNA and eIF3 (in case of mammalian eIF4G) and appears to constitute the primary ribosome recruitment

modulatory region (Preiss and Hentze, 2003). In mammals, the C terminal end region of eIF4G binds to the eIF4E-kinase Mnk-1 (Pyronnet et al. 1999). Phosphorylation of eIF4E at serine^{209} by Mnk-1 generally correlates with increased translation rates and serves as a marker of eIF4F integrity.

eIF4A, a DEAD-box RNA-dependent ATPase functions as an RNA helicase to unwind regions of secondary and tertiary structure in the 5'-ends of mRNAs using energy from ATP hydrolysis to disrupt RNA structure. eIF4B is a homodimer that can bind RNA by virtue of an N-terminal RNA recognition motif (RRM) and a C-terminal arginine-rich motif. It stimulates the RNA-unwinding activity of eIF4A (Gingras et al. 1999), probably by increasing the affinity of eIF4A for RNA (Abramson et al. 1988) and ATP (Bi et al. 2000). A second RNA-binding protein with similar functions, called eIF4H, has also been identified in mammals (Richter-Cook et al. 1998). Since the RNA-binding proteins that coat an mRNA must also be removed prior to 43S complex loading, it may be that eIF4F complex performs this task as well (Kapp and Lorsch, 2004). The eIF4G subunit of eIF4F might also aid in a similar function via its three RNA-binding sites, which are required for efficient translation initiation (Berset et al. 2003). Another RNA-dependent ATPase of the DEAD box family, Ded1p is known to unwind structures in the 5'-UTR of mRNA (Chuang et al. 1997). The giant multisubunit factor eIF3 may also play a role in mRNA loading due to its capacity to binds the 40S ribosomal subunit, the ternary complex and RNA (Chuang et al. 1997).

In addition to the events taking place at the 5'-end of the mRNA, events at the 3'-end of the mRNA may also take part in facilitating the loading of mRNA onto the 43S complex. The key players in these 3'-end mediated events are the 3'-poly(A) tail and the poly(A) binding protein (PABP). PABP interacts with eIF4G, and this interaction is thought to latch the mRNA by both ends, implying the potential to pseudo-circularise the mRNA, which in turn stimulates translation (Tarun and Sachs, 1996). This system provides a quality control mechanism. If the mRNA has been partially degraded and lost its 3'-end, it will not be translated efficiently. Thus, the system helps guard against the synthesis of truncated proteins that could be toxic to the cell (Kapp and Lorsch, 2004). Multiple molecules of PABP bind to the poly(A) tail, approximately one PABP per 27 nucleotides (Baer and Kornberg, 1983). Thus the poly(A) tail of an mRNA coated with PABP, forms a multivalent attachment site for eIF4G (Kapp and Lorsch, 2004). Studies have indicated that eIF4E's affinity for the 7-methylguanosine cap structure is enhanced by interaction between eIF4E and eIF4G,

and that the interaction between eIF4G and PABP increases eIF4E's cap affinity still further (Luo and Goss, 2001).

Initiation codon (AUG) selection:

After being assembled at the 5'-end of the mRNA, the 43S complex has to locate the appropriate start codon on the mRNA. The linear movement of the 43S complex along the 5' UTR of mRNA in search of an AUG triplet is termed 'scanning' (Kozak, 2002). Three players, eIF1, eIF2, and eIF5 appear to play pivotal role in initiation codon location, apart from the initiator tRNA. Each of the three initiation factors have been found in genetic screens affecting the fidelity of initiation codon selection (Kapp and Lorsch, 2004). In a reconstituted mammalian initiation system, toe-printing experiments have suggested that the 43S complex can assemble on the 5'-end of the mRNA in the absence of eIFs 1 and 1A, but will reach the initiation codon only upon addition of the two factors (Pestova et al. 1998). It has also been reported that the scanning process requires the hydrolysis of ATP (Kozak, 1980), although it is not clear what this energy is used for. One notion is that eIF4A and/or Ded1p may require ATP to unwind inhibitory structures in mRNA allowing the passive diffusion of 43S complex along the mRNA. When the ribosome randomly slides in the 3'direction over the unwound structures, they reform behind it, preventing backsliding. The diffusion based models are supported by data from reconstituted experiments which revealed that the 43S complexes can reach the AUG codon in an unstructured mRNA in the absence of ATP and eIF4A, however if the 5'-UTR is structured the two components are essential (Pestova and Kolupaeva, 2002). The exact role of eIF1 in initiation codon selection is not known but it may possibly interrogate the anticodon-mRNA base pairing and once perfect base pairing is achieved it may interact with the duplex and signal for termination in scanning. Later on it may also signal eIF2 and/or eIF5 to initiate GTP hydrolysis. Recently, it was determined that eIF1 bound to the 40S subunit at its P-site, and probably it alters the conformation of P-site in a way conducive for decoding (Pestova and Kolupaeva, 2002). eIF1 might also detect the formation of the codon-anticodon interaction indirectly, by interacting with the body of the initiator tRNA and sensing conformational changes in it when the initiation codon is reached.

The release of Met-tRNAi into the P-site upon selection of the initiation codon is performed by eIF2, by hydrolyzing GTP at appropriate time. Mutations that reduce initiation site selection fidelity have been isolated in all three subunits of eIF2.

Mutations in the γ subunit of eIF2 that affect initiation codon selection fidelity are in its GTP-binding site. These mutations appear to increase both the eIF5-indepedent GTPase activity of eIF2 and the rate of dissociation of Met-tRNAi from ternary complex (Huang et al. 1997). The constitutive activation of the GTPase activity of eIF2 would lead to premature release of Met-tRNAi. Hence the GTPase stimulatory activity of eIF2 (Das and Maitra, 2001) should be precisely regulated to synchronize with the AUG codon selection. The stimulation of eIF5's activity could be directly achieved by monitoring the codon-anticodon pairing, or it could be indirectly mediated through conformational changes in eIF1 or the 40S subunit. eIF5 interacts with Met-tRNAi indirectly via its direct interaction with the β-subunit of eIF2 and this interaction is critical for eIF5's function (Das and Maitra, 2001). Similar to mutations in eIF2, the mutations in eIF5 that reduce the fidelity of initiation site selection *in-vivo* appear to result in the hyperstimulation of GTPase activity of eIF2 (Huang et al. 1997).

While eIFs 1, 2, and 5 appear to be central to the AUG-recognition apparatus, evidence is mounting that other initiation factors may also play a role in the process. Mutations in eIF4G that weaken its interaction with eIF1 affect the initiation site selection fidelity (He et al. 2003). Since eIF3 interacts with eIFs 1, 2, and 5, it is possible that it may also play a role in locating the initiation codon (Phan et al, 1998). Experiments have established that in mammals the sequence context in which that AUG codons exist may also play a role in the selection of initiation site (Kozak, 1994). The consensus sequence in yeast and plants is AAAA**AUG**UCU and AA(A/C)A**AUG**GC, respectively and in mammals it is GCCACC**AUG**G (Kapp and Lorsch, 2004). *In-vitro* experiments have suggested that eIF1 might play a role in discriminating between AUGs in favourable and unfavourable contexts (Pestova and Kolupaeva 2002). An analysis of the human genome has indicated that as many as 40% of mRNAs have AUG codons upstream of the initiation site and the consensus sequence around the initiation codon is not as strongly conserved as proposed. This data suggests that the initiation of translation on many mammalian mRNAs may occur through unconventional pathways, such as internal ribosome entry or leaky scanning of upstream AUGs (Peri and Pandey 2001).

40S and 60S subunit joining: The recognition of initiation codon and deposition of the

Fig 1.1: **Overview of translation initiation step.**

The formation of eIF2.GTP.Met-tRNAi ternary complex is followed by the assembly of multifactorial complex (MFC) consisting of ternary complex, eIF3, eIF1 and eIF5. The MFC then joins the 40S ribosomal subunit generating a 43S pre-initiation complex. The mRNA bound at is 5' end by eIF4F complex (consisting of eIF4E, eIF4G and eIF4A) then joins the 43S pre-initiation complex forming 48S pre-initiation complex. The 48S complex then scans the mRNA for AUG codon. Selection of legitimate AUG codon is followed by a GTP hydrolysis event that results in the release of initiation factors and subsequent joining of 60S ribosomal subunit, forming the 80S initiation complex (Preiss and Hentze, 2003).

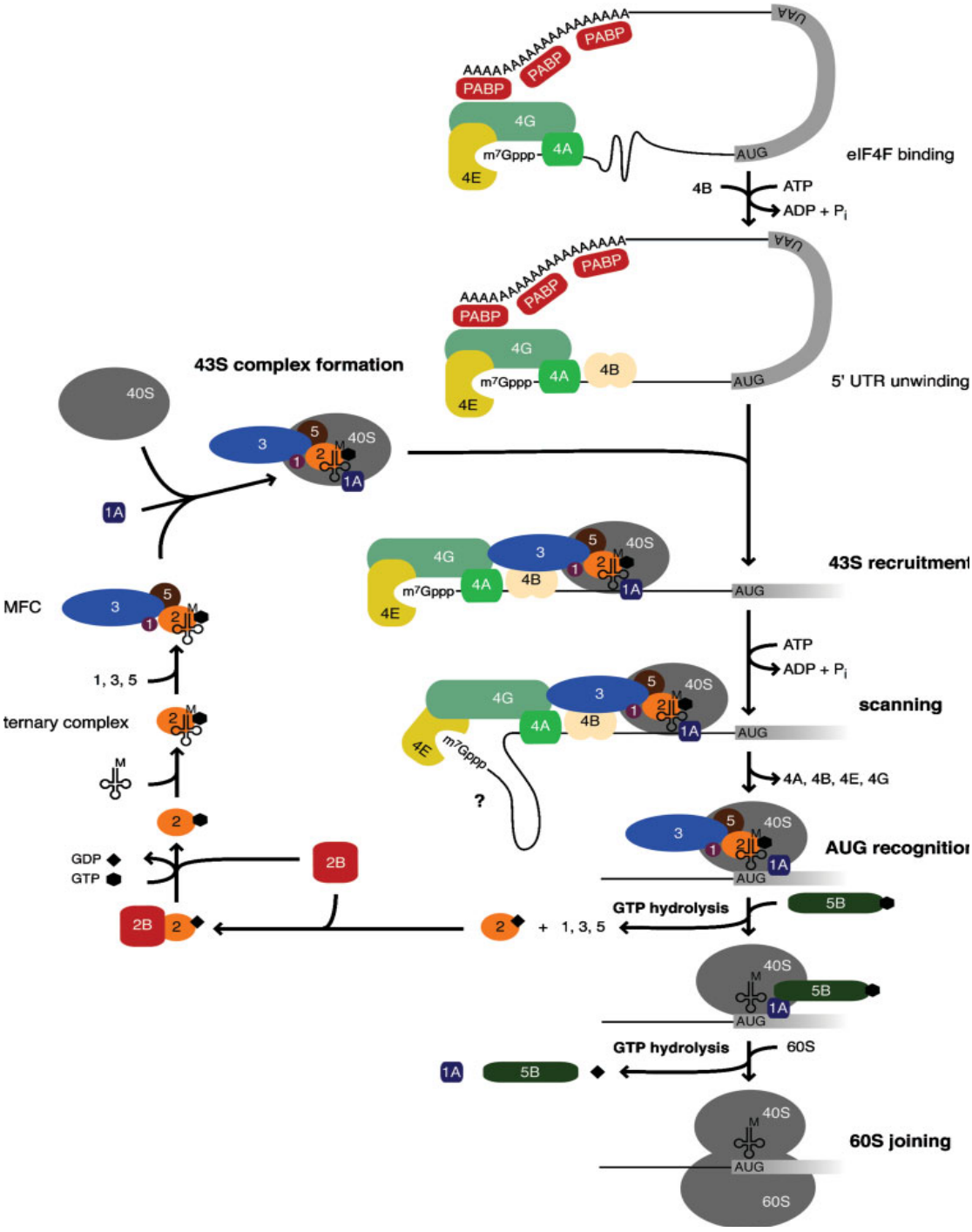
AAAAAAAAAAAAAAAAA
PABP
PABP
PABP
UAA
4G
4A
4E
m7Gppp
AUG
eIF4F binding
4B
ATP
ADP + Pi
43S complex formation
40S
3
5
2
M
1
1A
5' UTR unwinding
43S recruitment
MFC
1, 3, 5
ternary complex
scanning
4A, 4B, 4E, 4G
?
GDP
GTP
2B
AUG recognition
GTP hydrolysis
5B
+ 1, 3, 5
60S
60S joining

Fig 1.1

initiator tRNA into the P-site of 40S subunit is followed by the joining of the 60S subunit. The GTPase activating protein, eIF5 bound to the β-subunit of eIF2 is crucial for this step. The eIF5 activated GTP hydrolysis by eIF2 leads to the release of eIF2 and presumably the other initiation factors from the 48S complex making way for 60S subunit (Preiss and Hentze, 2003). Recently, *in-vitro* reconstitution experiments in yeast revealed that a second GTPase, eIF5B, is required for the 80S ribosome assembly (Pestova et al. 2000; Lee et al. 2002). GTPase activity of eIF5B is stimulated by 60S subunit and more strongly by the combination of 40S and 60S subunits. In its GTP-bound form, eIF5B has a high affinity for the ribosome and in its GDP-bound form, a low affinity (Kapp and Lorsch, 2004). Experiments with the GTPase-deficient eIF5B indicated that disruption of GTPase activity resulted in high frequency of failure to begin translation at upstream initiation codons and increased incidence of re-initiation downstream (Shin e al. 2002). This suggests that the timing of GTP hydrolysis is important to prevent premature release of eIF5B or premature dissociation of the Met-tRNAi (Kapp and Lorsch, 2004). In fact the release of initiation factors or the precise positioning of the initiator tRNA within the complex can act as signals for the promoting GTP hydrolysis. The joining of 60S subunit to the 48S complex also requires the dissociation of anti-association factor called eIF6. Other proteins such as eIF5A, eIF2A, eIF2C, and an ATP-binding protein ABC50 have also been implicated in translation however their precise roles are not yet determined (Kapp and Lorsch, 2004).

1.3.2: Elongation: Peptide elongation begins when the initiator tRNA is rightly placed in peptidyl tRNA site (P-site) of the 80S initiation complex with an empty A-site (aminoacylated tRNA site). Mammalian elongation cycle is primarily mediated by three eukaryotic elongation factors (eEFs): eEF1A, heterotrimeric eEF1B with three subunits α, β and γ, and eEF2. It basically involves; 1) the positioning of amino acylated tRNA at the A site, 2) formation of peptide bond between adjacent amino acids at P and A site and 3) the translocation of ribosome by three nucleotides along the mRNA. Eukaryotic elongation is mechanistically similar to that in prokaryotes however in contrast to prokaryotic factors eEFs undergo several posttranslational modifications (Kapp and Lorsch, 2004). An aminoacyl tRNA is carried to the A-site of the 80S complex as a ternary complex of eEF1A.GTP.aa-tRNA. Either cognate or noncognate aminoacyl tRNAs can bind to the ribosomal A site. Only the correct

codon-anticodon base pairing between the mRNA and the tRNA induces conformational changes such that eEF1A.GTP is brought into the GTPase activating center of the ribosome as shown in prokaryotes (Pape et al. 1998) so that the GTP is hydrolyzed and eEF1A.GDP is released. This ensures that only cognate tRNA is selected for entry into the polypeptide (Rodnina and Wintermeyer 2001). eEF1A.GDP must be recycled to its GTP-bound form so that it may participate in successive rounds of polypeptide elongation. The heterotrimeric protein eEF1B (eEF1Bα, β and γ) catalyzes this exchange. The nucleotide exchange activity is associated with the α and β –subunits but not with the γ–subunit. The mammalian eEF1B complexed with eEF1A is found associated with the endoplasmic reticulum (Sanders et al. 1996). The peptidyl transferase center of the ribosome catalyzes the formation of a peptide bond between the incoming amino acid and the peptide found at the P-site (Kapp and Lorsch, 2004). The above event results in a deacylated tRNA in a hybrid state with its acceptor end in the exit (E) site of the large ribosomal subunit and its anticodon end in the P site of the small subunit. The peptidyl-tRNA also occurs in a similar hybrid situation with its acceptor end in the P site of the large subunit and its anticodon end in the A site of the small subunit (Green and Noller, 1997). Eukaryotic elongation factor 2 (eEF2) accomplishes the tasks of pushing the deacylated tRNA and peptidyl tRNA completely into the E site and the P site respectively. It also ensures the translocation of mRNA by three nucleotides to place the next codon of the mRNA into the A site. eEF2 hydrolyzes GTP to facilitate the above processes (Wintermeyer et al. 2001). Once the translocation is completed, the ribosome will be ready to receive another aa-tRNA with the help of eEF1A and GTP. This cycle is repeated until a stop codon is encountered and the process of termination is initiated. The elongation factors function in translation by mimicking the anticodon stem loop of tRNA molecules (Nissen et al. 2000). An exception to the rule of evolutionary conservation of translation elongation is the existence of elongation factor 3 (eEF3) exclusively in fungi. The fungal eEF3 possesses ribosome-dependent ATPase and GTPase activities (Dasmahapatra and Chakraburtty, 1981). It facilitates the release of deacylated tRNA from E-site and enables efficient binding of the eEF1A.GTP.aa-tRNA ternary complex to the A-site (Triana-Alonso et al. 1997). Probably mammalian ribosomes actually possess an intrinsic eEF3-like stable ATPase and

GTPase activities (Rodnina et al. 1994). However, no homologue of fungal eEF3 has yet been identified in mammalian ribosomes.

1.3.3: Termination: The elongation cycle is repeated until a stop codon (UAA, UAG, or UGA) that does not have any cognate aa-tRNA is encountered and the process of termination of peptide chain elongation is initiated (Frolova et al. 1994). Termination or release factors aide the process of termination. Class I release factors identify the stop codon and facilitate the hydrolysis of the ester bond between nascent polypeptide and deacylated tRNA in the P-site by the peptidyl transferase center of ribosomes. Class II release factors are GTPases and stimulate class I release factor activity (Kapp and Lorsch, 2004). Eukaryotes have one release factor belonging to each of these classes (eRF1 and eRF3). The crystal structure of human eRF1 indicated that the protein has three domains in a structure reminiscent of the letter Y and as a whole resembles a tRNA molecule (Song et al. 2000). Sequence analysis of class I release factors revealed that the GGQ sequence motif is highly conserved in all species and mutations in this motif abolished the ability of human eRF1 to trigger peptidyl tRNA hydrolysis (Frolova, 1999). The GTPase function of eRF3 is thought to be involved in the dissociation of eRF1 and eRF3 complex from the ribosome (Frolova et al. 1996). Efficiency of translational termination is found to be modulated by the sequence context surrounding the termination codon (Pavlov et al. 1998).

1.3.4: Recyling: At the end of the termination the ribosome is left on the mRNA with a deacyclated tRNA. These ribosomal subunits have to be primed before they can take part in another round of initiation. Factors that increase the rate of dissociation of ribosomal subunits, mRNA, tRNA and sterically inhibit ribosome subunit association are required for ribosome recycling. Significant amount of data pertaining to bacterial recycling step is available. In bacteria, ribosome release factor (RRF), structurally resembling a tRNA recognizes the ribosome-mRNA-tRNA post-termination complex (Selmer et al. 1999). EF-G.GTP and IF3 then assist RRF in disassembling the post-termination complex (Karimi et al. 1999). RRF in conjunction with EF-G induces alteration in ribosome's structure destabilizing the ribosome subunit interface and interactions with tRNA and mRNA. IF3 then facilitates complete subunit dissociation and release of the tRNA and mRNA. Energy from GTP hydrolysis by EF-G could be used to actively facilitate these disruptions (Kapp and Lorsch, 2004).

The events and participants involved in ribosome recycling in eukaryotes are still elusive. Eukaryotes do not appear to have an RRF ortholog. eIF1A, eIF3, eIF1 and

eIF6 all have ribosome antiassociation activity *in-vitro* and may participate in ribosome recycling (Kapp and Lorsch, 2004). The closed-loop model of eukaryotic mRNAs suggests the possibility that termination and recycling may not release the 40S subunit back into the cytoplasm, and instead, the 40S subunit may be shuttled over the poly(A) tail back to the 5'-end of the mRNA mediated by the 5'and 3'-end associated factors. However this interesting and intriguing model is still unproven.

1.4: Regulation of translation: Translation of mRNA's is the final step in the gene expression pathway. Regulation of gene expression through modulation of translation is used to fine tune the levels and spatial distribution of proteins in several important cellular events like embryonic development, growth and differentiation, stress response and apoptosis (Clemens, 2001; Harding et al. 2000; Kuersten and Goodwin, 2003). It has been estimated that the fraction of genes devoted to translation may be as high as 35 to 45% (Sonenberg et al. 2000) and the modulation of this costly process can be used effectively to shift energy investment towards somatic maintenance. Two modes of translational control occur generally, a) global control in which the translation of most mRNAs is effected; and b) mRNA-specific control where the translation of a defined group of mRNAs is modulated without affecting general protein biosynthesis. Global regulation chiefly occurs by the modification of translation factors. The mRNA specific regulation may be mediated by regulatory protein complexes that recognize particular elements present in the 5′ and/or 3′ untranslated regions (UTRs) of the target mRNA and also by the local changes in the activity of general translation factors (Macdonald, 2001). An example of mRNA specific regulation is the local regulation of translation in a defined region of an oocyte or at the synaptic junctions of neuronal cells generating protein gradients that polarise the cell, or restrict protein expression to a defined region (Johnstone and Lasko, 2001).

To date, eIF2α, eIF2Bε, eIF4E, eIF4G, S6, eEF1 and eEF2 have been identified as targets for regulatory pathways, through post translational modifications such as phosphorylation (Rhoads, 1999). Cellular stresses also profoundly inhibit translation by modulating the efficiency of several eukaryotic translational factors (Patel et al. 2002). This stress response is highly conserved from yeast to mammalian cells (Wek et al. 2006) and is primary adaptive step aimed to ensure the conservation of resources like energy and amino acids that are needed to survive under adverse stress conditions

(Hinnebusch, 1994). Protein synthesis, is a multistep and multifactorial pathway and it is economical for the cell to control this kind of pathway at its start than to interrupt somewhere in the middle. Consistent with this logic, in most of the cases, translational regulation operates primarily at the initiation level rather than at elongation. However, in some rare cases the translational elongation is blocked, such as the signal recognition protein (SRP) mediated arrest of elongation during translocation of nascent polypeptide into the ER (Walter and Blobel 1981).

1.4.1: eIF2α phosphorylation and inhibition of eIF2B: Phosphorylation of the ser^{51} residue of eIF2α subunit is generally conceived as a stress signal. The eIF2α kinases contain unique regulatory elements that sense different sets of stress conditions and contribute to stress adaptation. Though the activity of these kinases is stimulated by distinct stress stimuli, they eventually converge upon eIF2α phosphorylation in mammalian cells (Wek et al. 2006). The serine/threonine eIF2α kinases are characterized by two distinct kinase activities: trans-autophosphorylation, and the phosphorylation of their substrate, eIF2α. Phosphorylation of eIF2α inhibits general translation through the inhibition of eIF2B, a heteropentameric guanine nucleotide exchange factor of eIF2 (Webb and Proud, 1997; Proud, 2005). At the end of initiation step, eIF2-GTP is hydrolysed to eIF2-GDP and Pi. The resulting eIF2-GDP complex subsequently leaves the ribosome. To return to its active GTP-bound state, eIF2 must undergo nucleotide exchange, and this function is performed by eIF2B. Modulation of the rate of GDP/GTP exchange is a key regulatory step for translation, both for overall rates of protein synthesis and for translation of certain specific mRNAs (Proud, 2005). Phosphorylated eIF2α has a higher affinity for eIF2B and it was hypothesized that eIF2α(P) forms a tight complex with eIF2B in which the guanine nucleotide exchange activity of eIF2B is inhibited (Rowlands et al. 1988). In fact, phosphorylation of ser^{51} residue of eIF2α has been shown to inhibit the GNE activity of eIF2B by promoting complex formation between eIF2α(P) and eIF2B (Sudhakar et al. 2000; 1999; Krishnamoorthy et al. 2001) as has been hypothesized earlier (Ramaiah et al. 1994; Rowlands et al. 1988). These events impair protein synthesis by preventing the recycling of eIF2 and reducing the availability of active ternary complex (eIF2.GTP.met-tRNAi) (Pavitt et al. 1998).

eIF2B is a complex of five non-identical subunits α, β, γ, δ and ε. The three homologous α, β and δ subunits act together forming a single regulatory domain

helping eIF2B to sense and respond to eIF2α phosphorylation at Ser^{51}(Kimball et al. 1998). Genetics and biochemical analysis revealed that eIF2Bε alone or in complex with other subunits possesses GEF activity. Comparing the activity of ε alone or in complex with the five subunits revealed that native complex formation enhanced its affinity for eIF2 and stimulated GEF activity 10–40-fold (Fabian et al. 1997; Williams et al. 2001; Pavitt et al.1998). Residues 518–580 of the ε subunit are well conserved and constitute the 'catalytic centre'. Findings suggest that eIF2B interacts with eIF2 via an interaction with eIF2β and then separate residues at the catalytic centre are important to mediate guanine nucleotide exchange. In yeast, eIF2Bγ is shown to enhance the activity of eIF2Bε leading to the idea that ε and γ subunits form the catalytic subcomplex (Pavitt et al. 1998; Pavitt, 2005).

The GNE activity of eIF2B is not only regulated by phosphorylation of eIF2α but also by its own phosphorylation. So far six phosphorylation sites have been identified in mammals (Wang et al. 2001). Two of the highly conserved sites are in the C-terminus of the ε-subunit and are required for the binding of eIF2B to eIF2. Additional two sites of phosphorylation are at the N-terminal of the catalytic domain (Wang et al. 2001). Phosphorylation of these N-terminal sites by glycogen synthase kinase 3 (GSK3) decreases the activity of eIF2B (Welsh et al. 1998). In the presence of insulin, GSK3 activity is inhibited and eIF2B is dephosphorylated making it more active (Pap and Cooper, 2002). In addition to insulin, growth factors, like NGF (neuronal growth factor) or EGF (epidermal growth factor) and amino acids have also been shown to influence eIF2B activity (Proud, 2005).

1.4.2: eIF2α kinases: The subfamily of eIF2α kinases includes the four well characterized kinases; General control non-derepressible-2 (GCN2), ds-RNA dependent eIF2α kinase (PKR), PKR like ER-resident eIF2α kinase (PERK) and Heme regulated inhibitor (HRI). These kinases share extensive homology in the kinase catalytic domains. However the regulation and regulatory domains of each of these kinases are very different.

HRI: Iron and heme play a pivotal role in hemoglobin synthesis and erythoid cell differentiation. In reticulocyte lysates iron or heme deficiency causes inhibition of protein synthesis, which can be rectified by supplementing with heme/iron (Kruh and Borsook, 1956; Waxman and Rabinovitz, 1966). Later it was discovered that the attenuation of translation was due to the activation of HRI (Chen and London, 1995).

Heme availability regulates HRI kinase. The HRI with one stable heme bound to the amino-terminal region occurs as a non-covalent homodimer with already autophosphorylated Ser and Thr residues in each subunit (Rafie-Kolpin et al. 2000). When heme is available in plenty, heme binds reversibly to the kinase insert region and promotes intersubunit disulphide bond formation of the homodimer and inhibits its autokinase and eIF2α kinase activity (Chen et al. 1989). In low concentrations of heme, the non-covalent homodimer undergoes further multiple autophosphorylation and phosphorylates eIF2α. Purified HRI binds ATP that is inhibited in the presence of heme, suggesting that the inhibition of ATP binding likely results in blocking of HRI activity. Heme displaces the free sulfhydryl groups required for ATP binding by promoting conformational change in HRI that brings essential sulfhydryl groups into close proximity for disulphide bond formation (Chen et al. 1989; Yang et al. 1992).

GCN2: In yeast, in response to amino acid starvation GCN2 is activated leading to eIF2α phosphorylation and inhibition of eIF2B (Hinnebusch, 1997). GCN2 contains a conserved N-terminal domain, highly charged psuedokinase domain, protein kinase domain, histidyl-tRNA synthetase-like region (HisRS) and a C-terminal domain (Hinnebusch, 2000). Uncharged tRNAs, that accumulate during amino acid starvation appear to be the activating ligands, because it has been shown that, even in amino acid rich conditions, abrogation of aminoacyl tRNA synthetase activity can stimulate GCN2 function (Wek et al, 1995; Hinnebusch, 1997). The C-terminal domain of GCN2 is associated with dimerization and ribosomal binding properties. Its motif preceding the HisRS domain interacts with the acceptor stem of tRNA (Wek et al, 1995; Zhu et al, 1996). In a hypothetical model, the inactive form of GCN2 is speculated to occur as a dimer, with the C-terminal domain inhibiting the autophosphorylation and substrate binding by interacting with the kinase domain. Binding of uncharged tRNAs to His-RS like domain induces conformational change that disrupts the interaction between C-terminal and kinase domain. Autophosphorylation ensures further structural alteration of the kinase domain facilitating substrate binding and phosphorylation (Hinnebusch, 2000).

Mammalian cells also respond to amino acid depletion by enhanced eIF2α phosphorylation (Sood et al. 2000). The first evidence that the mammalian homolog of GCN2 is also activated by deacylated tRNA is provided by a recent study in which liver from $GCN2^{+/+}$ and $GCN2^{-/-}$ mice were perfused in situ with medium containing

the histidinyl–tRNA synthetase inhibitor, histidinol. In liver of $GCN2^{+/+}$ mice, histidinol treatment resulted in enhanced phosphorylation of eIF2α as well as inhibition of eIF2B activity. In contrast, in the liver of $GCN2^{-/-}$ mice, histidinol had no effect on either eIF2α phosphorylation or eIF2B activity, suggesting that in mouse liver, a GCN2 kinase like homologue may be the predominant eIF2α kinase active in such conditions (Zhang et al. 2002).

PKR: Interferons (IFN) protect cells from viral infection, and among the molecules induced by IFN, is the dsRNA dependent eIF2α kinase, PKR. It is an enzyme with multiple effects in cells, and plays a critical role in the antiviral defense mechanism of the host (Garcia et al. 2006). PKR-triggered eIF-2α phosphorylation inhibits translation of viral mRNA. This constitutes the basic mechanism by which PKR exerts its antiviral activity on a wide spectrum of DNA and RNA viruses (Garcia et al. 2006). PKR has two dsRNA binding domains (dsRBD) in the N-terminus and a kinase domain (KD) near the C-terminus. PKR is activated in response to cellular, viral or synthetic dsRNA, such as viral dsRNA genomes, replication intermediates, mRNA transcripts with extensive secondary structure resembling dsRNA and poly IC (synthetic dsRNA). The dsRBDs wrap around the dsRNA molecule for optimal protein-RNA interactions, and hence the optimal length of dsRNA (~80 bp or longer) is required for an effective activation (Manche et al. 1992; Garcia et al. 2006). In nonstressed cells, PKR is in a monomeric latent state due to the autoinhibitory effect of its dsRBDs, which occlude the kinase domain. After binding dsRNA, PKR undergoes a number of conformational changes that relieve the autoinhibitory interactions of the enzyme and allow subsequent substrate recognition. Replacement of the dsRBD with an unrelated domain such as glutathione S-transferase, that is able to dimerize, constitutively activates PKR, both *in-vitro* and *in-vivo*, underscoring the importance of dimerization prior to activation (Ung et al. 2001). After homodimerization, PKR undergoes rapid autophosphorylation in a stretch of amino acids termed the activation segment. Among these, amino acids thr^{446} and thr^{451} are consistently phosphorylated during activation. Autophosphorylation further stabilizes PKR dimerization, and increases the catalytic activity of the kinase (Zhang et al. 2001). PKR activation is bimodal, low concentrations of dsRNA activate and high concentrations of dsRNA inhibit the activity. In addition to dsRNA, PKR can also be activated by polyanions like heparin *in-vitro* (Patel et al. 1994) and by caspase-3 *in-*

vitro (Saelens et al. 2001). PKR is associated to ribosomes, mainly to 40S subunits. Ribosomal association of PKR appears to be mediated by the dsRBDs (Garcia et al. 2006). PKR has also been detected in the nuclei of human and murine cells, specifically when the kinase is overexpressed (Jeffrey et al. 1995). The biological significance of PKR translocation to the nucleus is unknown, but recent data suggest that it could be involved in stress-induced apoptosis, since accumulation of phosphorylated PKR has been detected in tunicamycin-treated cultured cells and in neurons from patients with Alzheimer's disease (Onuki et al. 2004). Apart from sensing viral infections, PKR is activated by other activators, such as pro-inflammatory stimuli, growth factors, cytokines, and oxidative stress. Hence PKR integrates and transmits signals not only to the translational machinery but affects the activity of various factors involved in host defence, stress response and apoptosis, such as STAT, interferon regulatory factor 1 (IRF-1), p53, Jun N-terminal protein kinase (JNK), p38, and NF-κB (Garcia et al. 2006).

PERK: Physiological disturbances that perturb ER function lead to the accumulation of unfolded proteins in the ER lumen. These conditions activate an ER resident PKR like eIF2α kinase, PERK. Activation of PERK is primarily an adaptive measure and a part of coordinated stress response triggered by ER, known as the unfolded protein response (UPR). Activation of PERK attenuatates translation and halts the inflow of folding clients into the over burdened ER. Hence, PERK activation provides the ER with ample time to rectify the stress situation before translation is restored. PERK is a type 1 ER transmembrane protein with a regulatory luminal domain and catalytic cytoplasmic domain. In the nonstressed state, PERK is bound with BiP, an ER chaperone also known as GRP78 (glucose regulated protein 78). In response to accumulation of misfolded proteins during ER stress, BiP gets released from PERK to counter the increased load of unfolded proteins. Release of BiP from PERK unmasks the oligomerization motif in the luminal domain and facilitates its oligomerization and subsequent trans-autophosphorylation. Phosphorylation of serine and threonine residues in the activation loop of the kinase domain activates PERK and promotes eIF2α phosphorylation (Schroder and Kaufman 2005). Futher details on PERK and its role in UPR signaling are detailed below in the ER-stress signaling section.

1.4.3: Regulation by eIF4E: eIF4E, eIF4G, eIF4B and eIF3 that promote mRNA binding to ribosomes are phosphorylated and the phosphorylation status of these

Fig 1.2: **eIF2α phosphorylation and stress signalling.**

The eIF2α kinases, PKR, PERK, GCN2 and HRI sense diverse stress signals and converge on phosphorylation of eIF2α at ser^{51} position. Phosphorylated eIF2α, a stress signal attenuates general translation by inhibiting eIF2B. However the conditions of low availability of active ternary complex is shown to upregulate the translation of mRNAs with *cis*-acting elements in their 5' untranslated regions, like small upstream open reading frames, and internal ribosome entry sequence elements. One of the mRNAs upregulated in similar fashion in mammals is ATF4, a bZIP transcription factor integral to integrated stress response (Wek et al. 2006).

Fig 1.3: Regulation of eIF2B activity.

The eIF2B guanine nucleotide exchange activity is inhibited by the activation of eIF2α kinases that phosphorylate eIF2α. Phosphorylated eIF2 acts as a competitive inhibitor of eIF2B and sequesters it. Insulin signalling also modulates eIF2B activity by inhibiting GSK3β kinase. Mutations in eIF2B are implicated in vanishing white matter disease. Decline in eIF2B activity results in reduced recycling of eIF2.GDP to eIF2.GTP leading to a decline in the availability of active ternary complex (Pavitt, 2005).

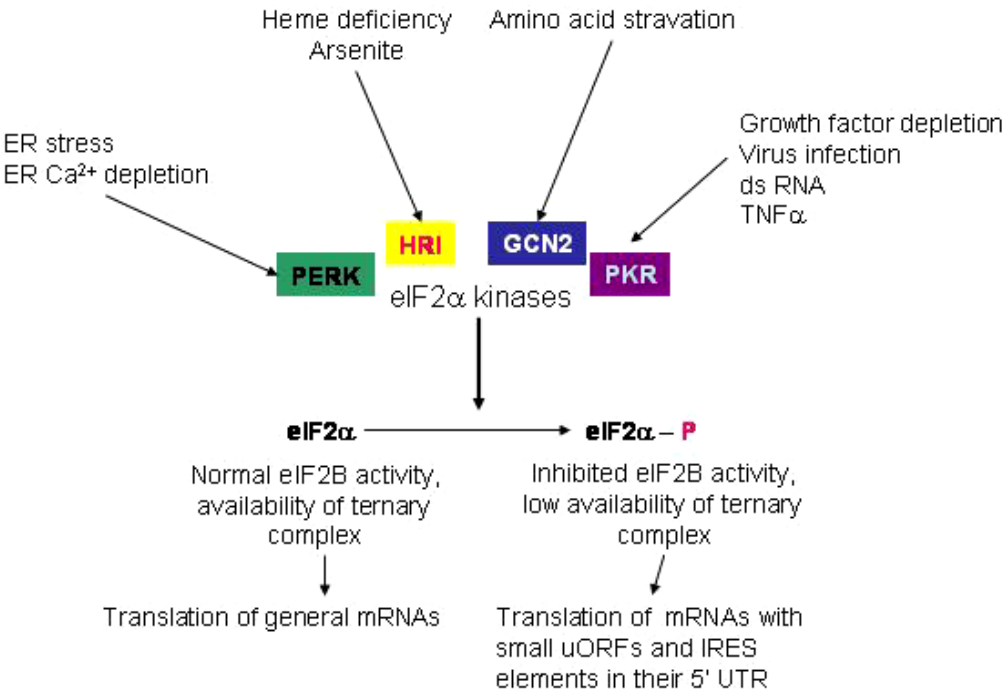
Heme deficiency
Arsenite
Amino acid stravation
ER stress
ER Ca2+ depletion
Growth factor depletion
Virus infection
ds RNA
TNFα
HRI
GCN2
PERK
PKR
eIF2α kinases
eIF2α
eIF2α – P
Normal eIF2B activity, availability of ternary complex
Inhibited eIF2B activity, low availability of ternary complex
Translation of general mRNAs
Translation of mRNAs with small uORFs and IRES elements in their 5' UTR

Fig 1.2

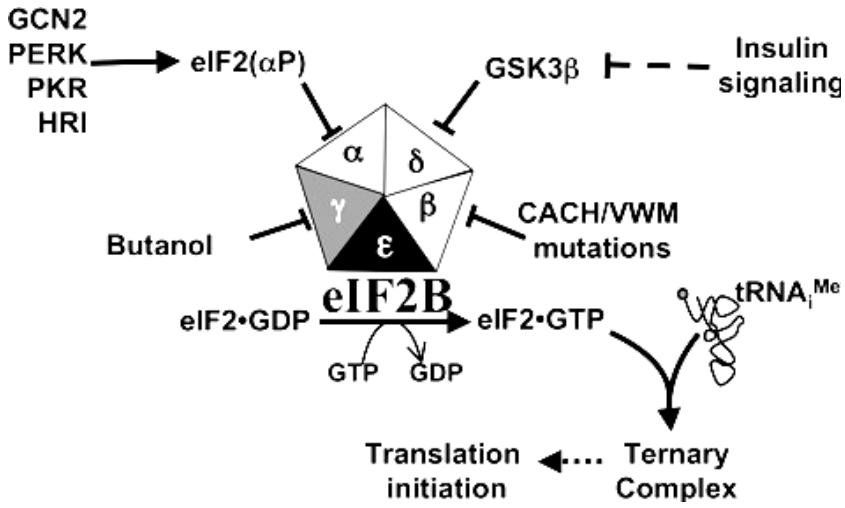
GCN2
PERK
PKR
HRI
eIF2(αP)
GSK3β
Insulin signaling
α
δ
γ
β
ε
Butanol
CACH/VWM mutations
eIF2B
eIF2•GDP
eIF2•GTP
tRNAiMe
GTP
GDP
Translation initiation
Ternary Complex

Fig 1.3

proteins correlates positively with both translation and growth rates of the cell (Rau et al. 1996). The best-studied and characterized factor among these is eIF4E, the cap binding protein. eIF4E appears to be a limiting translation initiation factor in most cell types, present at 0.01 - 0.2 molecules per ribosome, as compared to 0.5 – 3 molecules per ribosome, for other initiation factors (Hiremath et al. 1985). The eIF4E activity is regulated at multiple levels; (1) via modulation of its transcription, (2) by phosphorylation of the eIF4E protein, and (3) through its interaction with a family of translational repressor 4E binding proteins (4E-BPs). It is an important control point for the regulation of protein synthesis during cell division. Overexpression of eIF4E results in oncogenic transformation and elevated levels of eIF4E have also been observed in a variety of transformed cell lines and tumors (Lazaris-Karatzas et al. 1990; Miyagi et al. 1995). Overexpression of eIF4E helped to overcome the inhibitory influence of extensive secondary structure in synthetic 5' UTRs. It is proposed that under normal conditions, initiation on structured 5' UTRs is hampered and that upon activation of eIF4E more eIF4A (a RNA helicase) is recruited to mRNA cap binding eIF4F complex, resulting in unwinding of complex RNA secondary structures in the 5' UTR of mRNAs (Koromilas et al. 1992).

Activity of eIF4E is stimulated by its phosphorylation at ser^{209} and also by the phosphorylation of 4E-binding proteins (4E-BP's). eIF4E possess only a single phosphorylation site at ser^{209} (Joshi et al. 1995). A putative role for the ras/raf/MAPK(ERK) pathways is contemplated in eIF4E phosphorylation. Mitogen-stimulated pathway acting through the ERKs, and the stress-activated pathway acting through the p38 MAPKs, appear to converge at a common eIF4E kinase called Mnk1 (MAP kinase signal integrating kinase). PKC kinase is also shown to phosphorylate eIF4E *in-vitro* however its physiological significance is unclear. The MAP-kinases could by regulating eIF4E activity might play a role during embryonic development where specific classes of mRNAs need to be selectively translated (Raught and Gingras, 1999). *Drosophila*, expressing a Ser^{209}-Ala eIF4E mutant exhibits slow development and is small than wild type flies (Lachance et al. 2002). Mnk 1 does not interact directly with eIF4E but does interact with the eIF4G family of proteins (Pyronnet et al. 1999). The interaction between eIF4E and 4G is required for eIF4E phosphorylation because a mutant 4E that cannot interact with eIF4G is not efficiently phosphorylated (Pyronnet et al. 1999). Crystal structure of 4E predicted that phosphorylation at ser^{209} increases its affinity for 5'-cap of mRNA (Marcotrigiano et

al. 1997). However, recent studies contradict these predictions and suggest that phosphorylated 4E exhibits reduced affinity for cap analogs (Scheper et al. 2002). Evidence suggests that, ribosome associated eIF4E is more highly phosphorylated than free eIF4E (Pain, 1996).

1.4.4: Regulation by mTOR and PI3 kinase (phosphoinositide 3) signaling:

Protein synthesis is tightly regulated by cues that signal energy and nutrient levels. In many organisms, nutrient limitation has been found to inhibit translation, at least in part, by down-regulating the mTOR (mammalian target of rapamycin) kinase that plays a pivotal role for ribosomal biogenesis and translation itself (Wullschleger et al. 2006). Biological and genetic data provide evidence supporting the hypothesis that mTOR acts as a cellular sensor of nutrient availability. mTOR belongs to the phosphoinositide kinase-related kinase (PIKK) family of protein kinases, which contain a lipid kinase-like domain within their C-terminal region. Surprisingly, mTOR, like other PIKK family members, does not possess detectable lipid kinase activity, but instead functions as a Ser/Thr kinase (Tee and Blenis, 2005). A number of studies have implicated the mTOR signaling pathway in the stabilization of mRNAs, translational initiation and elongation, biogenesis of tRNA, and ribosomes. Specifically, mTOR kinase is known to influence the translational activation of TOP mRNAs (Meyuhas, 2000). TOP mRNAs contain tracts of oligopyrimidine (TOP) in their 5' untranslated regions. They encode many of the components of translational apparatus, including ribosomal proteins and elongation factors. Signaling through the mTOR pathway is controlled by growth factors, cellular energy levels, mitogens and nutrients. mTOR regulates protein synthesis through the phosphorylation of translation initiation factors such as 4E-BP1 (eIF4E-binding protein), eIF4B, eIF4G and the small ribosomal protein, S6 (Tee and Blenis, 2005). Thus the 5'-TOP regions in mRNAs confers growth and nutrient-dependent expression coordinating ribosomal genesis in response to nutrients and growth stimuli (Meyuhas, 2000). mTOR, possesses rapamycin-sensitivity, rapamycin hinders the ability of mTOR to form an effective multi-protein signaling complex with its downstream signaling targets such as 4E-BP1 and ribosomal protein S6 kinase 1 (S6K1) (Tee and Blenis, 2005). Although the precise mechanisms through which the mTOR kinase interacting proteins regulate the activity of the kinase have not been determined, recently two proteins, tuberous sclerosis complexes (TSC)-1 and -2 have been shown to repress mTOR signaling and there by suppress phosphorylation of its down stream targets 4E-

BP1 and S6K1 (Gao et a. 2002). Amino acids and insulin promote release of the TSC1-TSC2 complex from mTOR and permit PKB to phosphorylate the kinase on ser^{2448}.

1.4.5: eIF4E binding proteins (4E-BPs): eIF4E association with N-terminal of eIF4G depends on its interaction with 4E-BPs. Of the three known 4E-BPs (4E-BP1, 2, and 3), 4E-BP1 (also referred to as PHAS-I) is the best characterized. 4E-BP1 and eIF4G compete to bind on the same region on the surface of eIF4E. Hence, either 4E-BP1 or eIF4G can bind individually to eIF4E, but both cannot bind at the same time. Therefore, the interaction of 4E-BP1 with eIF4E blocks eIF4F complex formation by preventing the association of eIF4G (Tee and Blenis, 2005; Scheper and Proud, 2002). The affinity of 4E-BP1 for eIF4E is regulated by 4E-BP1 phosphorylation. 4E-BP1 phosphorylation occurs sequentially on at least seven sites (Thr37, -46, and -70 and Ser65, -82, -101, and -112, in humans). Hormones (insulin, angiotensin), growth factors (EGF, PDGF, NGF, IGFI, IGFII), cytokines (IL-3, GMCSF), mitogens (TPA), G-protein coupled receptor ligand (gastrin), amino acid availability, energy state of the cell and adenovirus infection have been shown to induce phosphorylation of 4E-BPs through PI3K and mTOR kinases (Raught and Gingras, 1999; Hay and Sonenberg, 2004). When mTOR activity is low, 4E-BP is hypophosphorylated allowing it to bind efficiently to eIF4E and block translation. Incontrast when mTOR activity increases, it phosphorylates 4E-BP, causing its affinity for eIF4E to drop and allowing cap-dependent translation to occur. Recently, several reports have indicated that 4E-BP activity is also regulated at the transcriptional level, 4E-BP transcription has been reported to be inhibited by the stress-dependent kinases ERK and p38 (Rolli-Derkinderen et al. 2003) and positively regulated by the forkhead transcription factor FOXO (Puig et al. 2003). 4E-BP expression is induced under various stress conditions including starvation. Through its regulation of overall cellular translation, 4E-BP may function as a “metabolic brake” conserving cellular energy. Although this brake may not be applied during normal development to limit growth, but it may be applied under stress conditions, including starvation, to control the animal’s metabolic rate and thus the rate at which it consumes its fat reserves. 4E-BPs role as a regulator of metabolism is substantiated by the fact that enhanced 4E-BP activity correlates with increased fat accumulation, whereas reduced 4E-BP activity leads to an increased rate of fat burn (Teleman et al. 2005).

1.4.6: S6 kinases (S6K): Small ribosomal subunit protein S6 is phosphorylated on multiple sites by the closely related kinases S6K 1 and 2. S6K's are activated by phosphorylation at multiple Ser/Thr residues by mTOR. Several signaling pathways such as PI3K, Ras/MAPK, and mTOR converge on S6K1 activation. Rapamycin or amino acid deprivation, however, leads to the inactivation of S6K1 even during conditions when PI3K and MAPK -mediated signaling is active suggesting that mTOR signaling primes S6K1 for inputs from other kinases (Tee and Blenis, 2005). mTOR may also suppress a phosphatase, as it has been observed that addition of rapamycin in cells leads to rapid dephosphorylation of the S6K1 multisite phosphorylation (Chung et al. 1992). Substrates of S6K1 other than S6, include the transcription factor CREMτ, the export factor CBP80 involved in RNA splicing, the apoptotic protein BAD, and eEF2 kinase (eEF2K) involved in translation control (Tee and Blenis, 2005). S6K1 may also play a role in mRNA biogenesis, as it is shown to phosphorylate SKAR (S6K1 Aly/REF-like target) a protein homologous to the ALY/REF protein family that are involved in coupling transcription, splicing, and mRNA nucleocytoplasmic export (Richardson et al. 2004). Earlier, S6K activation and S6 protein phosphorylation has been enhanced translation of TOP mRNAs. However, later it was shown that rapamycin which completely inhibits S6K activity only moderately effected TOP mRNA translation (Stolovich et al. 2002). More recently, it has been reported that TOP mRNA translation is still active in embryonic stem cells with a disruption of both S6K1 and S6K2 alleles (Pende et al. 2004). The combined knockout of S6K1 and S6K2 revealed the presence of another S6K that may be insensitive to rapamycin (Pende et al. 2004).

Recent studies implicate growth-factor regulated PI3 kinase (PI3K) pathway in controlling TOP mRNA translation (Hamilton et al. 2006). PI3K activates its downstream effector kinase PKB (protein kinase B) via PDK1 (phosphoinositide-dependent kinase 1). Specific inhibitors of PI3K completely blocked the growth factor dependent translational activation of TOP mRNAs, whereas the expression of constitutive active forms PI3K or PKB relieved the translational block imposed on TOP mRNAs (Hamilton et al. 2006). Although the precise role of S6K or PI3K/mTOR pathway in TOP mRNA translation has not been clearly defined as yet, it is quite evident that S6K1 and 4EBP1/eIF4E function cooperatively in translational

Fig 1.4: **Regulation of eIF4E.**

eIF4E activity is modulated by phosphorylation on its ser^{209} residue by Mnk kinase. Activation of Mnk that occurs in response to growth stimuli and stress is mediated by ERK and p38 MAPK respectively (Sonenberg et al. 2000).

Fig 1.5: **mTOR and PI3 kinase signalling pathways in translational regulation.** Nutrient and growth stimuli regulate the activity of several translational factors via the activation of PI3 and mTOR kinases. The brief details of the signalling mechanisms are detailed in the text (Wullschleger et al. 2006).

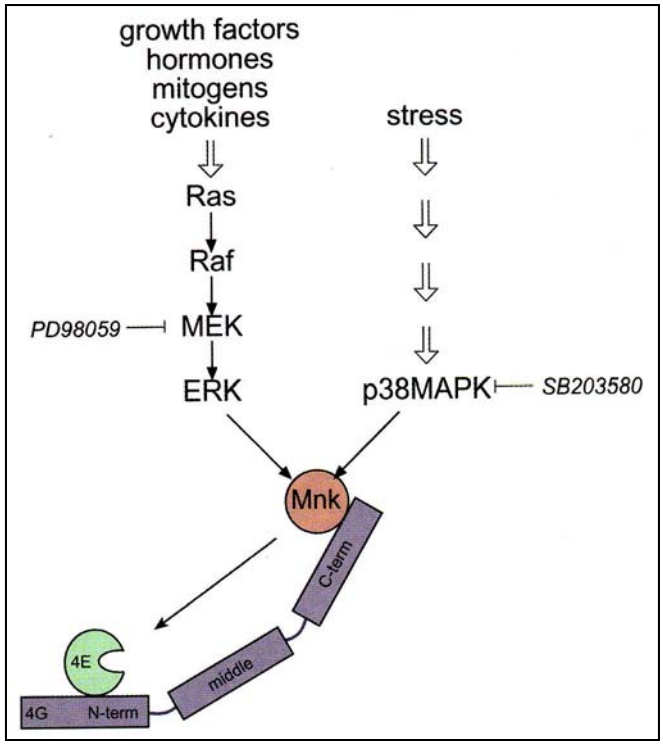
growth factors
hormones
mitogens
cytokines
stress
Ras
Raf
PD98059
MEK
ERK
p38MAPK
SB203580
Mnk
C-term
4E
middle
4G
N-term

Fig 1.4

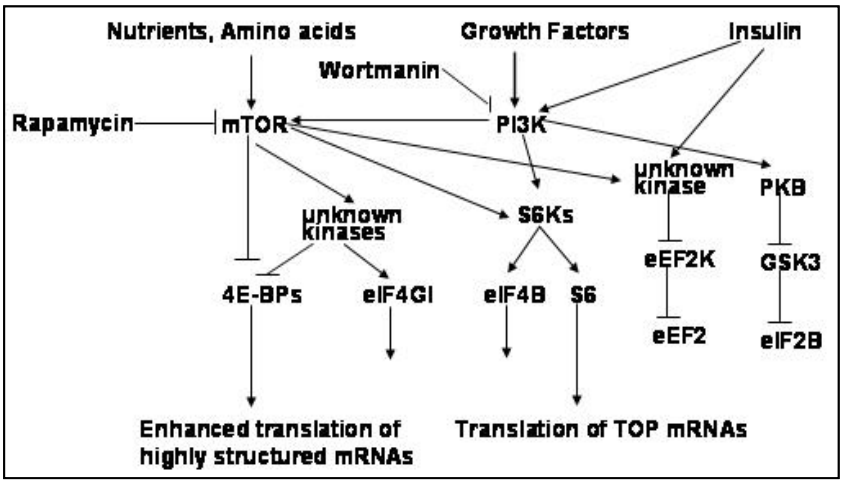
Nutrients, Amino acids
Growth Factors
Insulin
Wortmanin
Rapamycin
mTOR
PI3K
unknown kinase
PKB
unknown kinases
S6Ks
eEF2K
GSK3
4E-BPs
eIF4GI
eIF4B
S6
eEF2
eIF2B
Enhanced translation of highly structured mRNAs
Translation of TOP mRNAs

Fig 1.5

control to regulate both mammalian cell growth, size and proliferation (Fingar et al. 2002).

1.4.7: Phosphorylation of eIF4G and eIF4B: Both eIF4G and eIF4B are phosphoproteins, however the physiological significance of the phosphorylation on their activity remains to be elucidated. Although the signaling mechanisms that effect eIF4G phosphorylation are unknown two distinct sets of phosphorylation sites have been identified in the protein. One set is located at the C-terminus in the hinge region and the other at the N-terminus. Phosphorylation of ser^{1108}, ser^{1148}, ser^{1192} residues in the hinge region of eIF4GI is elevated by serum or insulin treatment, and inhibited by rapamycin or PI3K inhibitors (Raught et al. 2000). Interestingly, these residues are not phosphorylated by PI3K, mTOR, and S6K's *in-vitro*. eIF4G is shown to have similar affinity for its binding partners even if it is phosphorylated or not. It is speculated that the phosphorylation could effect changes in eIF4GI structure to increase eIF4GI activity toward rapamycin-sensitive mRNAs (Raught et al. 2000). Increased binding of eIF4E to eIF4G favours translation of mRNAs encoding cell-cycle and growth-regulated proteins. Cleavage of eIF4G by proteases is shown to facilitate the translation of house keeping proteins whereas the translation of proteins involved in cell cycle and growth are inhibited (Keiper et al. 1999).

Mammalian eIF4B occurs as multiple isoforms and eIF4B hyperphosphorylation has been observed upon treatment of cells with serum, insulin, or phorbol esters. eIF4B can be phosphorylated *in-vitro* with S6K1, PKC, PKA, CKI, CKII and PAKI. Although, PI3K and mTOR appear to signal eIF4B phosphorylation, it appears that it is a direct target of S6K1 *in-vivo* (Keiper et al. 1999).

1.4.8: Other routes regulating translational initiation: Apart from the canonical mechanisms involving translational factors, structural features (*cis* acting elements in 5' and 3' UTR of mRNAs), spatial distribution of mRNAs, and ligands that prevent or facilitate the assembly of 48S initiation complex also modulate translation. For example, small RNA molecules of ~22 nucleotides in length that are known as micro RNAs (miRNAs), have emerged in recent years as mRNA-specific regulators of translation. miRNAs hybridize by incomplete base-pairing, usually to several sites in the 3′ UTR of target mRNAs. Because the target mRNA remains intact after miRNA binding, the miRNAs are believed to repress translation, rather than prevent translation by degrading the mRNA. The first miRNAs known to play role in translational regulation are *lin-4* and *let-7*, which are crucial for regulating the

developmental timing in *C. elegans* (Carrington and Ambros, 2003). Recent studies have shown that *miR*122 represses translation of arginine/lysine transporter CAT-1 mRNA and enriches its localization in the cytoplasmic mRNP granules known as P body. The P bodies are believed to be mRNA storage and/or degradation sites. Under conditions of stress such as amino acid deprivation, the mRNA re-enters active translation, concomitant with the loss of target mRNA from P bodies. These findings substantiate the hypothesis that the aggregation of mRNAs into large mRNP complexes (such as P bodies or other cytoplasmic foci), is an important mechanism in mRNA translational repression, and mRNA metabolism (Baker and Collar, 2006).

The modification within a ribosome may also regulate translation. Chemical modifications of ribosomal proteins or rRNA, or changes in the association of ribosomal or nonribosomal proteins, lead to adjustments in the affinity of the ribosome for particular classes of mRNAs. Preliminary observations, have led to the idea that posttranslational modification of the ribosome fine tunes the binding of non-ribosomal proteins and, in turn, modulates the affinity of the ribosome for specific mRNAs (Baker and Collar, 2006).

Cis-acting elements like the iron-responsive element (IRE) if present in the 5' UTR or 3' UTR of mRNAs can also affect translation. When iron is limiting, cytoplasmic iron-regulatory proteins (IREP's) regulate the translation of ferritin and transferrin mRNA's by interacting with the IRE present in their 5' UTR or 3' end respectively. The interaction with IREP's is shown to inhibit the cap-dependent recruitment of ferritin mRNA to the 43S preinitiation complex (Muckenthaler et al. 1998), and increase the stability of tranferrin mRNA by sterically interfering its accessibility to endonucleases (Binder et al. 1994).

Other *cis*-acting elements like internal ribosome entry sites (IRESs) can direct translation via a cap-independent mechanism, allowing for direct recruitment of ribosomes to the initiation codon. Initially discovered in the 5' untranslated regions of picornavirus RNAs, IRES elements have also been detected in cellular mRNAs as well (Johannes et al. 1999). It is now widely accepted that IRES in cellular mRNAs maintain synthesis of key survival proteins under conditions of stress, when cap-dependent initiation is compromised. However, the details of many specific RNA structures and proteins involved in IRES function still remain obscure.

Some translational regulators that function during embryonic development target the formation of the eIF4F complex. The cytoplasmic polyadenylation element-binding

protein (CPEB) regulates the translation of maternal mRNA during vertebrate oocyte maturation and early development. This protein binds to a cytoplasmic polyadenylation element (CPE) located in the 3' UTR of target mRNAs. CPEB protein promotes both silencing of the mRNA before oocyte maturation as well as subsequent cytoplasmic polyadenylation and translational activation (Mendez and Richter, 2001). To repress translation, CPEB binds a protein known as maskin that contains an eIF4E-binding domain. As such, the CPEB–maskin complex functions as 4E-BP and competes with eIF4G for binding to eIF4E (Stebbins-Boaz et al. 1999). Translational activation by CPEB occurs via its phosphorylation on ser^{174} residue by Eg2 kinase that leads to displacement of poly(A) ribonuclease from the multiprotein mRNP, thereby facilitating poly(A) addition catalyzed by another complex member, Gld-2, a noncanonical poly(A) polymerase. The poly(A) tail and the specificity of this polyadenylation has been ascribed to the translational activation of specific maternal mRNAs (Baker and Collar, 2006).

During anteroposterior axis formation in the early *D. melanogaster* embryo, the mRNA that encodes the posterior determinant nanos becomes concentrated and is specifically translated at the posterior pole of the oocyte. The protein smaug binds to the 3' UTR of unlocalized nanos mRNA and represses its translation by recruiting the eIF4E-binding repressor protein cup31. Cup is also recruited by a RNA binding protein, bruno, to the oskar mRNA that encodes the posterior determinant, thereby preventing oskar synthesis during the transit of oskar mRNA from the anterior to the posterior pole of the oocyte (Wilhelm et al. 2003; Nakamura et al. 2004). In contrast, another protein, bicoid, the anterior determinant, inhibits the translation of caudal mRNA at the anterior pole, by directly binding to eIF4E (Niessing et al. 2002).

1.4.9: Regulation of elongation:

Inhibition of elongation would temporarily serve to reduce the amount of energy, when the cell needs to conserve energy or divert it to other processes. Several instances are known where elongation of protein synthesis is regulated. For example, the signal recognition protein (SRP) mediates the arrest of elongation of mRNAs encoding secretory proteins. As the nascent polypeptide is directed into the ER lumen, the docking protein relieves the elongation block restoring protein synthesis (Walter and Blobel 1981). Insulin has been shown to increase rapidly the rate of elongation, as measured from ribosomal transit time in several cell types (Chang and Traugh, 1997). The α and β-subunits of eEF1B and eEF1A serve as substrates for PKC *in-vitro* and

undergo phosphorylation in response to phorbol esters *in-vivo* (Venema et al. 1991). Phosphorylation of eEF1B complex increases its gunanine nucleotide exchange activity and its affinity for charged tRNAs (Redpath et al. 1996). Phosphorylation of eEF2 at thr^{56} by eEF2 kinase, a calcium/calmodulin dependent protein kinase, interferes with the binding of eEF2 to the ribosome and thus impairs the translocation step (Browne and Proud, 2002). Treatment with serum, bradykinin, thrombin and glutamate, that enhance cytoplasmic Ca^{2+} levels results in increased eEF2 phosphorylation (Redpath et al. 1996; Palfrey et al. 1987). It is likely that mTOR also regulates the rates of peptide elongation through eEF2 phosphorylation by modulating the activity of eEF2 kinase. mTOR may function to reduce the rates of elongation when nutrients and energy levels become depleted. ADP ribosylation of eEF2 catalyzed by diphtheria and pseudomonas A toxins also inhibits eEF2 activity (Fendrick et al. 1992).

1.4.10: Physiological significance of eIF2α phosphorylation: Four known eIF2α kinases, PKR, PERK, HRI and GCN2 converge upon the phosphorylation of ser^{51} residue in eIF2α in response to varied stress stimuli and elicit a stress-responsive gene expression programme designed to alleviate cellular damage or alternatively induced apoptosis. The eIF2α kinases that are activated under varied stress conditions such as suboptimal levels of amino acids, glucose or serum, heat shock, heavy metal stress, redox imbalance, oxidative stress, hypoxia and endoplasmic reticulum stress, help the cell to sense and adapt to various stress condition. In addition to inhibiting general protein synthesis, elevated levels of phosphorylated eIF2α mediates gene-specific translational upregulation of transcription factors that initiate stress-responsive signaling cascades such as redox regulation, aminoacid metabolism, and in some cases also initiate apoptosis (Wek et al. 2006; Hinnebusch, 1994; Clemens, 2001; Samuel, 1993; Harding et al. 2000). The mechanism of transcript-specific translational upregulation by phosphorylated eIF2α is partly dependent on the upstream open reading frames (uORFs) present in the 5' untranslated region (UTR) of mRNA. Transcripts upregulated in this manner are ATF4, ATF3 and CAT1 in mammalian cells and GCN4 in yeast (Wek et al. 2006; Harding et al. 2000; Hinnebusch, 1994). Yeast GCN4 mRNA has four short upstream open reading frames (uORF1-4). In normal physiological conditions, when eIF2B is active, the small ribosomal subunits are recharged rapidly with ternary complex after translation of

uORF1 making it possible for them to efficiently reinitiate at uORF4. Translation of uORF4 results in dissociation and release of ribosomes because of GC-rich sequence surrounding the uORF4 stop codon. Therefore only few recharged small ribosomal subunits reach the GCN4 main ORF resulting in its low expression. Under conditions of amino acid depletion, GCN2 kinase phosphorylates eIF2α that inhibits eIF2B resulting in low availability of active ternary complex. This physiologic condition declines the efficiency of small ribosomal subunits in scanning and reinitiating at the uORFs and increases the number of ribosomes that continue to scan to the main ORF of GCN4 resulting in a paradoxical induction of GCN4 translation. GCN4, a transcription factor can in turn induce genes involved in amino acid biosynthesis and related metabolic pathways (Hinnebusch, 1997). Similarly, in mammalian cells an identical mechanism involving multiple short uORFs upregulates the translation ATF4 mRNA when eIF2α is phosphorylated. ATF4 is a bZIP transcription factor integral to the integrated stress response (ISR) and induces genes involved in amino acids transport, glutathione biosynthesis and oxidative stress response (Harding et al. 2003). The mechanism mediated by uORFs appears to upregulate the translation of genes with critical biological functions specifically when general translation is inhibited. The rapidly increasing list of genes containing uORFs that are probably upregulated by eIF2α phosphorylation includes the CCAAT/enhancer binding proteins α and β; oncoprotein mouse double-minute 2; human epidermal growth factor receptor-2; S-adenosylmethionine decarboxylase; and β2-adrenergic, retinoic acid, glucocorticoid, and estrogen receptors (Jefferson and Kimball, 2004). In addition some mRNAs with IRES elements in their 5'UTR are also efficiently translated when eIF2α is phosphorylated (Gerlitz et al. 2002; Gebauer and Hentze, 2004). A number of cellular mRNAs such as vascular endothelial growth factor, hypoxia-inducible factor-1α, protein kinase C-δ, basic fibroblast growth factor, platelet derived growth factor (PDGF), c-myc, X-linked inhibitor of apoptosis (XIAP), cationic amino acid transporter 1 (CAT1) and ornithine decarboxylase contain IRES elements. Accumulating evidence suggests that, eIF2α phosphorylation mediated transcript-specific-translational upregulation is important for normal physiological development, differentiation, stress response, adaptation and cell death (Gerlitz et al. 2002; Scheuner et al 2001; Jefferson and Kimball, 2004; Brewer et al. 1999; Harding et al. 2000).

Several sudies projected both prosurvival and proapoptotic nature of eIF2α phosphorylation. For example, expression of the eIF2α [S51A] non phosphorylatable mutant can suppress apoptosis (Srivastava et al.. 1998) whereas activation of PKR through FADD or TNFα pathway can induce, or enhance, apoptosis (Der et al. 1997; Balchandran et al. 1998; Scheuner et al. 2006). In fact, recently it was shown that expression of phosphomimetic eIFα [S51D] mutant is sufficient to activate caspase-3 and induce apoptosis (Scheuner et al. 2006). Although it is unclear how PKR induced eIF2α phosphorylation mediates apoptosis, it is likely that other potential substrates of PKR have an influential role. Proapoptotic transcription factor CHOP (C/EBP homologous protein) induced via the eIF2α-ATF4 pathway can also probably induce apoptosis when eIF2α is phosphorylated (Marciniak et al. 2003). It is also pertinent to mention that translational attenuation rapidly brings down the levels of short lived anti-apoptotic proteins like IAPs (inhibitors of apoptosis) giving the proapoptotic proteins an upperhand (Scheuner et al 2006).

Paradoxically eIF2α phosphorylation is also shown to protect cells from ER-stress induced apoptosis and viral infection. The protective effects of eIF2α phosphorylation in ER stress may be mediated by decline in client protein load on the overburdened ER and/or by reprogramming the induction of eIF2α–specific stress responsive genes that ensure restoration of ER homeostasis (Boyce et al 2005; Harding et al. 2001). Repression of a constitutive eIF2α–specific phosphatase (CReP) or ER-stress induced eIF2α phosphatase offered cytoprotection against ER stress, viral infection and heat shock (Boyce et al. 2005; Jouse et al. 2003). Pre-emptive conditional phosphorylation of eIF2α also protected cells from lethal effects of oxidants, peroynitrite donors and ER stress (Lu et al. 2004). Interestingly severe or prolonged ER-stress can also lead to apoptosis and CHOP is shown to play a significant role in this process (Schroder and Kaufman, 2005).

The paradoxical effects of PERK or PKR mediated eIF2α phosphorylation can be visualized because of the other potential targets of these kinases and/or by the spatial distribution of the targeted eIF2α in the cell. However, to understand the discrepancies involved in these ambiguous outcomes several clarification still remain to be elucidated.

eIF2α phosphorylation is also implicated in NF-κB activation, autophagy, hypoxic

tumor survival and memory. Although the exact mechanism is unclear, NFκB activation mediated by eIF2α phosphorylation primarily involves the repression of IκBα translation (Deng et al. 2005). NF-κB plays a predominant role in inflammatory responses and immune development. ER stress stimuli such as nutrient deprivation and viral infection converge on eIF2α phosphorylation and also stimulate the eIF2α-kinase-dependent autophagy, an important conserved mechanism that involves bulk degradation of cellular contents by autophagolysosomes as an adaptation to environmental stress (Tallóczy et al. 2002). NRF2, a substrate of the eIF2α kinase, PERK and ATF4, a product of eIF2α phosphorylation are shown to offer protection to tumors in hypoxic microenvironment by inducing an antioxidant gene expression programme (Cullinan and Diehl, 2006). The eIF2α-ATF4 pathway is also shown to bidirectionally mediate the switch from short term to long term synaptic plasticity and memory. Mutations that decrease eIF2α phosphorylation led to enhanced long-term memory, whereas treatment with a drug that prevents eIF2α dephosphorylation impaired long-term memory (Mattioli et al. 2007). From the above studies it is quite evident the eIF2α phosphorylation and global translational control play a lynchpin role in several important cellular physiological processes.

1.5: Age-associated changes in translation:

The rate of protein synthesis undergoes detrimental changes in many organisms, organs and tissues during aging (Kimball et al. 1992; Rattan and Clark 1996; van Remmen et al. 1995; Cales et al. 1986). In this context, two important questions arise, i) what are the molecular mechanisms underlying the changes in protein synthesis during aging? ii) whether these alterations a consequence of aging or the cause for aging?

Studies on various components of the protein synthetic machinery during cellular aging have revealed a decline in the efficiency and accuracy of ribosomes, an increase in the levels of rRNA and tRNA (Rattan, 1996). In rats, the levels of eIF2 and the activity of its guanine nucleotide exchange factor eIF2B have been shown to decline during development and aging, proportional to the decline in the rate of protein synthesis (Cales et al. 1986; Kimball et al. 1992; Garcia et al. 1994; Vargas and Castaneda, 1984). In the rats it was shown that the levels of eIF2B declined in adult rat liver relative to that in developing rat liver, although no significant alteration in the phosphorylation status of eIF2 is observed (Garcia et al. 1994). Interestingly, even

though the levels of eIF2 protein diminished with age, no parallel decline in the eIF2 mRNA levels is observed (Kimball, 1992). Other study has shown that the levels of eIF2 and phosphorylation status fairly remained constant in suckling and adult rats however the amount of eIF2 associated with ribosome declined with age (Martin et al. 1993). In developing mice hippocampus, the levels of eIF2B were shown to decline from embryonic day 18 suggesting that regulated protein synthesis is highly active in young developing plastic brain (Inamura et al. 2003). In contrast, no difference in methionyl-tRNA synthetase activity is observed during development (Cales et al 1986).

Regulation at the elongation level is also shown to play a role in age related modulation of protein biosynthesis (Gabius et al. 1983; Vargas and Castaneda 1981; Jager et al. 2002). In rodent liver and brain, it is observed that the level and activity of eEF1α decline as a function of age (Rattan, 1991, 1992). A decline of active eEF2 by over 60% is reported during *in-vitro* aging of human fibroblasts (Riis et al, 1990), although no difference in the specific activity of eEF2 is observed with aging in rat and mouse liver (Takahashi et al. 1985).

Aging does not always lead to decline in protein synthesis, and rates of general or specific protein synthesis depending on tissue type may decrease, remain unchanged, or increase. For example, upon the onset of sarcopenia or loss of muscle mass, the old rats make a futile attempt to maintain muscle mass by increasing general proteins synthesis (Kimball et al. 2004). The above various observations signify the multifactorial etiology of translational modulation during aging and development in animal tissues. And it remains to be determined if it is the altered activity of the translation factors rather than quantity important for the regulation of protein synthesis during aging (Rattan, 1991). Detailed studies on the structure and function of ribosomes, activities and levels, and the mode of regulation of all initiation and elongation factors during the entire life span are required to have a clear cut idea of the role of protein biosynthesis during aging.

Nutrient and energy cues effectively alter protein synthesis rates. Protein synthesis is a key regulated cellular process that links nutrients availability to an organism's growth and development. Major genetic modulators of life span such as insulin/IGF-1 and mTOR signaling pathways that influence aging in varied organisms from yeast to rodents modulate protein biosynthesis (Sonenberg et al. 2000; Shamji et al. 2003;

Kapahi et al. 2004; Kenyon, 2005). In lower eukaryotes several studies have effectively shown that diverting cellular energy to stress repair and maintenance by reducing protein synthesis extended life span considerably. Translation is energy consuming complex process, with 35 to 45% percent of total genes devoted translation and inhibiting the costly process is likely to shift the energy investment towards somatic maintenance. The TOR kinase that regulates cell growth in response to favorable nutrient conditions and/or growth-factor signals via its influence on cytoplasmic translation (Schmelzle and Hall, 2000), has attracted attention from the aging-research community due to its apparent influence on life span in a number of organisms. Decreased TOR signaling has been shown to extend life span in the nematode *C. elegans* (Vellai et al. 2003). Overexpression of a dominant-negative allele of TOR or inhibitors of TOR extends life span in *Drosophila* (Kapahi et al. 2004). And deletion of the TOR1 gene is shown to increase replicative life span in *S. cerevisiae* (Kaeberlein et al. 2005). Infact high-throughput screen for gene deletions that extend chronological life span yielded a number of genes that are influenced by TOR pathway and are involved in nutrient sensing (Powers et al. 2006). Unlike earlier claims linking TOR inhibition to induction of stress-responsive genes, recently it was shown that the deletion of TOR1 delays aging by increasing mitochondrial respiration via enhanced translation of mtDNA-encoded oxidative phosphorylation complex subunits. This may lead subsequent increase in mitochondrial oxygen consumption and decline in ROS generation (Bonawitz et al. 2007). Reduced expression of genes involved in translation such as ribosomal proteins, translation initiation factors, and ribosomal protein S6 knase, that results in reduced rate of protein synthesis increased the life span in *C. elegans* (Pan et al. 2007; Hansen et al. 2007). Similarly, deteriorating ribosomal efficiency by deletion of ribosomal protein genes also enhanced the replicative life span of *S. cervisiae* (Kaeberlein et al. 2005). Although it is still not clear as to how lowered rates of protein synthesis increased life span in the lower eukaryotes, recently it was shown in *C. elegans* that these perturbations increased heat resistance, suggesting that reducing protein synthesis resulted in increased energy expenditure in somatic maintenance (Hansen et al. 2007). It is also suggested that the general reduction of protein synthesis, due to the decreased frequency of mRNA translation, also lowers the cellular load of erroneously synthesized polypeptides (Hipkiss, 2007).

In mammals also over expression of eIF4E is shown to enhance senescence suggesting that the hypothesis connecting energy conservation to life span extension can hold good even in higher eukaryotes (Ruggero et al., 2004). In such case one would expect an inverse correlation between rate of protein synthesis and longevity in all species. However since the implications of this detrimental change in protein synthesis are manifold, and leads to reduced maintenance, repair, and metabolic functioning of the cell (Rattan, 1996), the significance of the above hypothesis in mammals can only be ascertained after determining as to what extent of decrease in general or specific protein synthesis can be beneficial in life span extension.

1.6: Stress response and aging:

Survival of an organism is a function of maintenance and repair capacities. Abilities such as DNA repair, anti-oxidant defence, stress response, adaptation, proliferation and turnover of cells correlate positively with life span of the species and there is a negative correlation between longevity and the rate of damage accumulation such as mutations, epimutations, macromolecular oxidation, and aggregation (Rattan, 1989). However it is imperative to mention that, is it the failure of maintenance and repair mechanisms that manifests into aging or is it the response to the damage per se that leads to the phenotype of aging (Sierra, 2006).

Aging is associated with changes in gene expression. Interestingly stress-responsive genes show induction during aging. In the neocortex of aging mouse, approximately 20% of genes upregulated during aging link to immune or inflammatory and stress responses (Prolla, 2002). Similarly, a report cites that 16% of genes showing twofold increase with age belong to stress response (Weindruch et al. 2001). The enhanced expression of stress responsive genes during aging suggests that aging is associated with concomitant increase in stress.

In contrast, genes responsible for metabolic and biosynthetic processes are down regulated in gastrocnemius muscle and heart of mice as a function of age (Edwards et al. 2004; Weindruch et al. 2001). With age a decline in mitochondrial function, turnover and ATP generation is evident by decline in genes such as ATP synthase-A chain, NADP transhydrogenase, LON protease, and ERV1 responsible for mitochondrial bioenergetics; biogenesis; and function (Beckman and Ames 1998; Miyoshi et al 2006; Shigenaga et al. 1994). Mitochondrial sarcomeric creatine kinase is highly induced in aged animals possibly as compensation to the increased production of reactive oxygen species (ROS) in mitochondria of aged animals

(Stachowiak et al. 1998; Sohal and Weindruch 1996). Also the efficiency of signal transduction pathways activated by oxidative stress declines with age (Holbrook and Ikeyama 2002, Yoon et al. 2002) and very recently this hypothesis was substantiated by a study which showed a decline in the fidelity of anti-oxidant defence system with age in leydig cells of rats (Luo et al. 2006). The decline in antioxidant defence system may lead to an excessive cumulative oxidative damage incurred by functionally important macromolecules, especially proteins with age (Stadman, 2006; van der Vlies, 2003).

Calcium homeostasis is essential for cellular stress signaling. However recent studies suggest it declines with age. For example, increased activity of voltage activated calcium channels in the central nervous system of aged animals (Disterhoft et al. 1994); enhanced expression of dihydropyridine-sensitive L-type calcium channel and reduced turnover of ryanodine receptor and Ca^{2+}ATPase in aged Fishcer rats suggests a decline in calcium homeostasis with aging (Ferrington et al. 1998). With age accumulation of abnormal proteins increases that can arise due random errors during protein synthesis, spontaneous or metabolite-induced modifications of amino acid side chains and changes in polypeptide folding. An important function in a cell controlling the accumulation of abnormal proteins is their degradation by proteases. Aging is shown to be associated with reduced expression of genes involved in protein turnover, such as the 20S proteasome subunit, the 26S proteasome component TBP1, ubiquitin-thiolesterase and the Unp ubiquitin-specific protease (Schwartz and Ciechanover 1999; Rattan, 1996). Aberrantly modified proteins are better protease substrates than their normal counterparts. In spite of this sensitivity to proteolysis these modified proteins accumulate during aging. This indicates a drop in the activity of proteases that degrade abnormal proteins during senescence (Rosenberger, 1991).

Hence in aged individuals, a decline in translational efficiency (Kimball et al. 1992) coupled with inefficient intra-cellular signaling, deregulated calcium homeostasis, damaged macromolecules (specifically proteins), reduced ATP generation, and protein turnover, may compromise the rapid and effective stress responsive measures. Taken as a whole, these results provide evidence that, although the aging process is characterized by the activation of an adaptive stress response, a concomitant decline in other important cellular functions ceases the ability of an aged individual to effectively respond and adapt to the stress. For example, aging has been related to a decline in stress response (Finkel and Holbrook, 2000) and in the ability to induce

heat shock proteins (HSP's, especially HSP 70) during stress (Richardson and Holbrook, 1996). Procedures that enhance stress tolerance such as preconditioning by mild stress stimulus can induce hormesis and enhance longevity (Soti and Csermely 2003; Rattan 2004).

1.6.1: Endoplasmic Reticulum (ER) stress: ER is an organelle involved in secretory or membrane protein biosynthesis, folding and trafficking. The protein processing is handled by energy consuming ER resident chaperones, foldases and carbohydrate processing enzymes. To ensue the proper function of its resident proteins, the ER maintains a highly oxidative and calcium rich environment. Physiological disturbance like nutrient deficiency, reduced generation of ATP, deregulated calcium homeostasis, redox imbalance, oxidative stress, elevated protein synthesis, and viral infection, can perturb the protein processing by the ER and lead to accumulation of unfolded protein in the ER. In order to restore homeostasis, the ER elicits a coordinated and intricate adaptive signaling cascade, called the **unfolded protein response** (UPR) (Schroder and Kaufman, 2005). Since the maintenance of the highly sensitive ER luminal environment and availability of energy is a perquisite for ensuing proper processing of proteins in ER, the ER is gaining recognition as a cellular stress sensor and lynchpin in the stress response. The ER couples the physiological stresses prevalent in the cell to its function and thus integrates the UPR to the cellular stress response. Survival of an organism is a function of maintenance and repair capacity, hence the efficient function of ER is highly essential to decelerate the aging process. It is highly possible that the ER-stress response declines during aging, since aging is associated with decline in ATP generation, deregulated calcium homeostasis, redox imbalance, oxidative damage to important molecules of ER, coupled with decline in protein degradation and turnover (Miyoshi et al. 2006; Ferrington et al. 1998; Holbrook and Ikeyama, 2002; van der Vlies et al. 2003; Rattan, 1996). Since age-associated alterations in cellular environment facilitate ER dysfunction, it is very likely that the aged cells are under the influence of incessant ER stress. There are models which suggest that ER stress may be a link between oxidative damage of proteins and the aging process (Ermak & Davies, 2002; van der Vlies et al. 2003). ER dysfunction is speculated to be one of common denominator for cell injury in several neurodegenerative disorders that generally associate with aging (Paschen, 2003). Also, there is direct evidence that gadd153 expression is elevated in the liver with aging and

this sensitizes aged liver cells to oxidative stress (Ikeyama et al. 2003; Li & Holbrook, 2004).

UPR is an evolutionarily conserved mechanism that maintains cellular homeostasis. In lower eukaryotes it is less complex and primarily involves an adaptive mechanism. However in mammals the UPR is more diverse and complex and can also trigger apoptosis if the adaptation fails. Three ER transmembrane proteins, IRE-1 (inositol-requiring and ER to nucleus signaling), ATF6 (activated transcription factor) and PERK, that serve as ER-stress transducers facilitate the transduction of ER-stress signal to the cytoplasm and nucleus. IRE-1 is present in yeast but other two are present only in mammals (Tirasophon et al. 1998; Haze et al. 1999; Shi et al. 1998; Harding et al.1999). In normal conditions the ER-resident chaperone BiP is bound to the luminal domains of the three ER-stress transducers. The release of BiP is a prerequisite for their activation and over-expression of BiP suppresses the ER-stress response (Hendershot, 2004; Bertolotti et al. 2000). Hence BiP is considered to be an ER stress sensor and a critical regulator of ER stress response. The ER stress-induced adaptive signaling cascade includes two arms: translational attenuation followed by transcriptional induction of ER chaperones, components of ER-associated degradation (ERAD) machinery and antioxidant genes.

IRE-1: Mammalian IRE-1 is a type-1 ER transmembrane bifunctional glycoprotein with serine/threonine kinase and endoribonuclease activities in its cytosolic domain (Tirasophon et al. 1998). Two isoforms, α and β, have been identified in mammals. IRE-1α is ubiquitously expressed whereas β expression is limited to gut epithelial cells (Wang et al. 1998; Bertolotti et al. 2001). In response to accumulation of unfolded proteins BiP bound to the luminal domain of IRE-1 is released promoting IRE-1 dimerization, autophosphorylation and subsequent activation of its endoribonuclease and kinase activity (Wang et al. 1998; Bertolotti et al. 2000). Active endoribonuclease splices XBP-1 (X-Box binding protein) mRNA in mammals (Yoshida et al. 2001) and HAC1 (homologous to ATF/CREB1) mRNA in yeast (Schroder and Kaufman, 2005). The splicing events result in the formation of a 376 amino acid XBP-1 protein, with a novel C terminus that acts as a potent basic leucine zipper transcription factor, in contrast to a 261-aminoacid XBP-1 protein encoded by unspliced XBP-1 mRNA (Yoshida et al. 2001). The bZIP transcription factor encoded by spliced XBP-1 mRNA translocates to the nucleus and induces genes encoding ER

chaperones, components of ERAD and those involved in lipid biosynthesis (Schroder and Kaufman, 2005; 2006; Yoshida, 2007).

PERK: is an ER-resident eIF2α kinase, it resembles in structure to IRE-1α, however it lacks the endoribonuclease activity (Harding et al. 1999). In response to accumulation of unfolded proteins in ER, PERK is also activated by a mechanism similar to IRE-1. Activation of PERK attenuates general translation through eIF2α phosphorylation, reducing the inflow of folding clients into the overburdened ER (Schroder and Kaufman, 2005; 2006). PERK also phosphorylates Nrf2 (Nuclear Factor-E2 related factor2), a transcription factor that induces genes involved in anti-oxidation, detoxification of enzymes, immune signaling, protein trafficking, protein degradation, chaperones, cell growth and survival (Cullinan and Diehl, 2006). Like in all instances of eIF2α phosphorylation, PERK mediated eIF2α phosphorylation also upregulates the translation of transcripts with multiple short uORF, like ATF4. ATF4 a bZIP transcription factor that inturn induces ER chaperone genes like BiP, and those involved in amino acid transport, glutathione biosynthesis, and redox regulation (Harding et al. 2003.)

ATF6: In contrast to IRE-1α and PERK, ATF6 is a type-2 transmembrane protein with N-terminus in the cytoplasm and C-terminus in the ER lumen (Schroder and Kaufman, 2005). The mechanism of activation of ATF6 in response to accumulation of unfolded proteins is also different from that of IRE-1and PERK. The luminal domain of ATF6 carries golgi localization signals that are masked by BiP in unstressed conditions. BiP release facilitates ATF6 translocation to golgi were it is sequentially processed by S1P and S2P proteases to yield an active 50 kDa p50ATF6 transcription factor with high trans-activating potential (Schroder and Kaufman, 2005). The first cleavage by S1P occurs in the luminal domain of golgi on the serine protease 1 site of ATF6 and the second cleavage, called regulated intramembrane proteolysis, by S2P occurs in the transmembrane region (Yoshida, 2007). Under-glycosylated ATF6 monomers can be efficiently transported to golgi, whereas the transport of glycosylated ATF6 dimers is inhibited (Nadanaka et al. 2006; Hong et al. 2004). Genes induced by p50ATF6 include ER chaperones, Herp (hyperhomocysteinemia–induced ER stress responsive protein) and protein disulfide isomerase (Schroder and Kaufman, 2005).

Among the adaptive responses, translational attenuation mediated by the PERK pathway appears to be the immediate event, followed by transcriptional induction of ER stress responsive genes. Evidence however suggests that it is not necessary all adaptive pathways are invoked in tandem but activation of a subset of pathways can occur. For example, IRE-1, but not the PERK pathway, is activated in the maturation of B cells (Wu and Kaufman, 2006). UPR is shown to play a significant role in the embryonic development and in maturation of secretory cell types such as antibody-producing plasma cells, osteoblasts that secrete collagen, and insulin-secreting pancreatic β cells. It also plays a role in liver development, sleep deprivation, aging and also promotes tumor viability in hypoxic environments (Wu and Kaufman, 2006; Zhao and Ackerman, 2006).

1.6.2: ER stress and Apoptosis: In mammalian cells an incessant ER stress beyond the limits of adaptation some how bolsters the proapoptotic potential of UPR paralleled by a subsequent decline in the adaptive measures. The suicide of unhealthy cells via apoptosis is a general phenomenon in multicellular organisms to clear the non-functional cells. Interestingly, the ER stress induced apoptosis is mediated both by mitochondria (intrinsic pathway) and/or through activation of proapoptotic downstream kinases of the extrinsic pathways.

Role of IRE-1: IRE-1-XBP-1 is the last pro-survival branch of the UPR (Szegezdi et al. 2006). However persistent stress triggers the pro-apoptotic pathway of IRE-1 (Wang et al. 1998; Szegezdi et al. 2006). Although the exact mechanisms are unclear, IRE-1 interacting proteins like JIK (c-JUN-N-terminal inhibitory kinase) and JAB1 (Jun activation domain-binding protein 1) may play a role in switching the IRE-1 signaling from that of prosurvival to proapoptotic nature. The proapoptotic effects of IRE-1 are generally mediated by its cytoplasmic interacting partner TRAF2 (tumor necrosis factor receptor-associated factor 2). Activation and dimerization of IRE-1 facilitates the recruitment and clustering of TRAF2 (Urano et al. 2000). The kinase adaptor TRAF2 recruits and activates the apoptosis–signal-regulating kinase (ASK1) (Nishitoh et al. 1998). The heterotrimeric complex of IRE-1-TRAF2-ASK1 leads to the activation of ASK's downstream targets MKK3 and 6, and MKK4 and 7 (Urano et al. 2000; Nishitoh et al. 1998). MKK3 and 6 activate p38 MAP kinase whereas MKK4 and 7 activate JNK (c-Jun-N-terminal kinase) (Roux and Blenis, 2004). The IRE-1 ineracting partner JIK appears to facilitate IRE-1-TRAF2 interaction whereas

Fig 1.6: **Unfolded protein response (UPR) and adaptive signalling mechanisms.** Physiological disturbances like, nutrient deprivation, ATP depletion, calcium fluxes, redox imbalance, viral infection, etc, perturb ER function and lead to the accumulation of unfolded proteins in the ER lumen. To restore homeostasis, the ER evokes a stress response pathway known as unfolded protein response (UPR). In mammals three ER transmembrane proteins IRE-1, PERK and ATF6 transduce the ER-stress signal to cytoplasm and nucleus. In normal conditions the stress transducers are held inactive by the binding of BiP to their ER luminal domains. When unfolded proteins accumulate in the ER, BiP bound to the transducers gets released to taper the increased demand. BiP release is followed by the subsequent oligomerization, autophosphorylation of IRE-1 and PERK. ATF6 activation proceeds via a different mechanism and involves its translocation to golgi where it gets processed by golgi resident S1P and S2P proteases. Active IRE-1 splices XBP1 mRNA and the spliced XBP1 mRNA generates a potent bZIP transcription factor that translocates to nucleus and upregulates transcription of genes that encode chaperones and those involved in lipid synthesis and ER associated degradation (ERAD). Active PERK phosphorylates eIF2□ and attenuates general translation, reducing the ER client protein load. The physiological condition of reduced availability of active ternary complex (eIF2.GTP.Met-tRNAi) upregulates the translation of ATF4, a potent bZIP transcription factor that induces CHOP and genes involved in redox regulation, and amino acid metabolism. Processed p50ATF6 also translocates to nucleus and upregulates ER chaperone and ERAD genes. The unfolded protein response primarily constitutes an adaptive response, however persistent or irreversible ER stress may trigger its proapoptotic potential (Schroder and Kaufman, 2005).

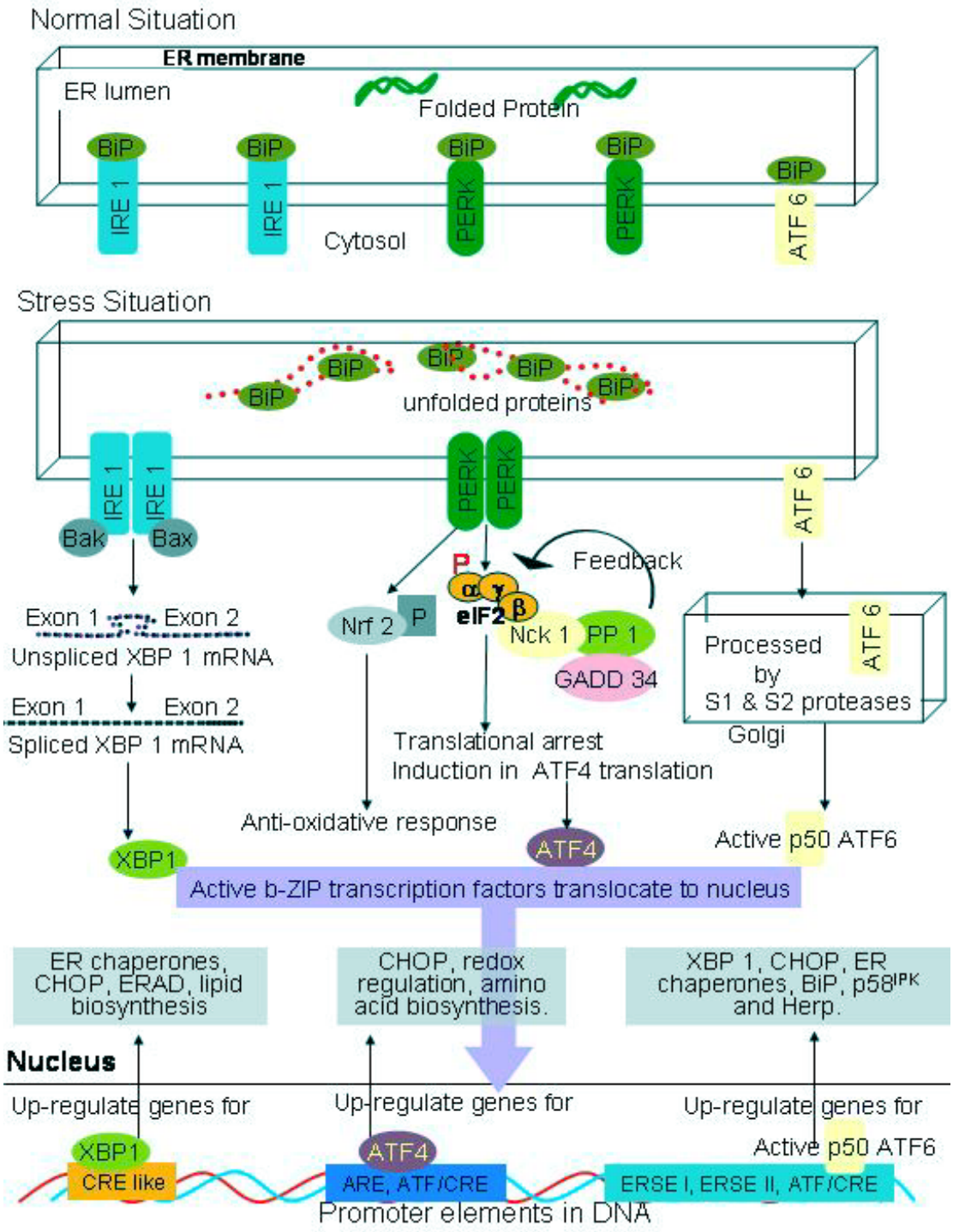
Normal Situation
ER membrane
ER lumen
Folded Protein
BiP
IRE 1
PERK
ATF 6
Cytosol
Stress Situation
unfolded proteins
Bak
Bax
Feedback
eIF2
Nrf 2
P
Nck 1
PP 1
GADD 34
Exon 1
Exon 2
Unspliced XBP 1 mRNA
Spliced XBP 1 mRNA
Processed by S1 & S2 proteases
Golgi
Translational arrest
Induction in ATF4 translation
Anti-oxidative response
Active p50 ATF6
XBP1
ATF4
Active b-ZIP transcription factors translocate to nucleus
ER chaperones, CHOP, ERAD, lipid biosynthesis
CHOP, redox regulation, amino acid biosynthesis.
XBP 1, CHOP, ER chaperones, BiP, p58IPK and Herp.
Nucleus
Up-regulate genes for
CRE like
ARE, ATF/CRE
ERSE I, ERSE II, ATF/CRE
Promoter elements in DNA

Fig 1.6

JAB1 impedes the interaction (Szegezdi et al. 2006). However, in severe ER stress IRE-1-JAB1 interaction is diminished facilitating IRE-1 interaction with TRAF2-ASK1 (Szegezdi et al. 2006). Active JNK phosphorylates and activates the proapoptotic transcription factor c-Jun, promoting apoptosis. The precise role of p38 kinase cascade in the above process is still unknown. IRE-$1^{-/-}$ fibroblasts are defective in apoptosis mediated by JNK activation and similarly $ASK^{-/-}$ neurons resist ER stress-induced apoptosis, whereas over-expression of ASK, sensitizes the cells to apoptosis (Urano et al. 2000; Szegezdi et al. 2006; Yoshida, 2007). The IRE-1-JNK pathway is also linked to the activation of c-Abl, an ER-associated tyrosine kinase that translocates to mitochondria during ER stress and affects the release of cytochrome *c* (Yoshida, 2007). In ER stress, IRE-1-TRAF2 interaction is also implicated in the activation of procaspase-12 (Wang et al. 1998; Yoneda et al. 2001). Calpains, the Ca^{2+} dependent proteases also activate procaspase-12 upon ER Ca^{2+} release (Szegezdi et al. 2006; Yoshida, 2007). IRE-1 expression is shown to trigger caspase-12 activation and apoptosis (Wang et al. 1998). In addition, caspase-7 is also shown translocate to ER and promotes the activation of caspase-12 during ER stress (Szegezdi et al 2006; Yoshida, 2007). In rodents, the ER localized caspase-12 is possibly a key mediator in ER stress-mediated apoptosis.

Role of CHOP: CHOP/GADD153 (Growth arrest and DNA damage inducible gene 153) is primarily proapoptotic and among the highest inducible genes during ER stress. Activation of all the three arms of UPR (IRE-1, ATF6 and PERK) is required for the maximal induction of CHOP because its promoter is regulated by four *cis*-acting elements that can be transactivated by transcription factors, ATF4, ATF6 and XBP-1 and also by ATF2 and ATF3 (Oyadomari and Mori, 2004). CHOP induction is highly diminished in cells lacking PERK or expressing the non-phosphorylatable [S51A] eIF2α mutant, suggesting that PERK-eIF2α pathway plays a dominant role in CHOP induction during ER stress (Harding et al. 2000). As CHOP is downstream to ATF4-eIF2α phosphorylation, activation of other eIF2α kinases can also lead to CHOP induction. CHOP can heterodimerize with other bZIP transcription factors such as, C/EBPs, ATF3, and ATF4, and can act as a transcriptional repressor or an activator (Oyadomari and Mori, 2004). In many ER stress models, induction or over-expression of CHOP sensitized cells to ER stress-induced apoptosis, whereas CHOP deletion offers protection (Oyadomari and Mori, 2004; Marciniak and Ron, 2006).

CHOP induces the transcription of proapoptotic proteins such as GADD34 (cofactor of eIF2α phosphatase), ERO1 (endoplasmic reticulum oxidoreductin 1), DR5 (death receptor –5), carbonic anhydrase VI and TRB3 (Tribbles related protein-3) (Yoshida, 2007; Szegezdi et al. 2006, Marciniak and Ron, 2006). The pro-apoptotic effects of CHOP in ER stress may be mediated by the induction of GADD34, a cofactor of eIF2α phosphatase that dephosphorylates eIF2α (Marciniak and Ron, 2006). Translational restoration in stressed and overburdened ER aggravates ER stress by enhancing the folding client load. Alternatively, during persistent ER-stress the translation of mRNA's coding for proapoptotic proteins may be restored by GADD34 induction, leading to apoptosis (Szegezdi et al. 2006). GADD34 null mice resist ER stress–mediated nephrotoxicity induced by tunicamycin, like the CHOP null animals (Marciniak and Ron, 2006). In fact another notion is that, GADD34 mediated translational restoration may be required for adaptive cell survival measures. This notion is supported by a study in which GADD34 mutations have been shown to sensitize cells to ER stressor thapsigargin (Marciniak and Ron, 2006). In addition, over-expression of CHOP is also shown to offer protection in mouse models of Pelizaeus-Merzbacher leukodystrophy. These findings raise the possibility that CHOP-GADD34 signaling pathway has a dual role, protecting from and promoting cell death (Marciniak and Ron, 2006). Other targets of CHOP like ERO1α, DR5, and carbonic anhydrase VI, may probably play a more significant role in the apoptotic process than GADD34. ERO1α oxidizes PDI that inturn transfers the oxidizing equivalents to nascent folding proteins promoting disulfide bond formation. This oxidative protein folding process eventually involves the transfer of electrons to molecular oxygen, hence CHOP/ERO1α induction may result in enhanced generation of ROS further aggravating ER-stress and promoting cell death (Tu and Weissman, 2004, Marciniak and Ron, 2006). The lethality of CHOP-ERO1α pathway may be more pronounced in cells with elevated protein synthesis. DR5 and carbonic anhydrase VI induce apoptosis by activating caspases and affecting changes in cellular hydrogen ion concentration respectively (Yoshida, 2007; Ron and Marciniak, 2006). CHOP induced TRB3 protein promotes apoptosis by interacting and inhibiting the prosurvival Akt kinase (Szegezdi et al. 2006). The transactivation ability of the proapoptotic transcription factor CHOP is increased upon p38 mediated phosphorylation of its ser^{78} and ser^{81} residues (Maytin et al. 2001; Szegezdi et al.

2006). The proapoptotic and prosurvival effects of CHOP may also be modulated by its interacting partners. For instance, cells deficient in C/EBPβ, a dimerization partner of CHOP are resistant to ER-stress induced apoptosis (Oyadomari and Mori, 2004; Zinszner et al. 1998).

Role of BCL2 protein family: Since mitochondria are juxtaposed to ER, in many cases ER stress is communicated to mitochondria triggering apoptosis (Hacki et al. 2000; Oakes et al. 2006; Szegezdi et al. 2006). The communication of ER-stress signal to mitochondria is generally accomplished by release of ER-Ca^{2+} and its uptake by the mitochondria. Some proteins of the BCL2 family, known to regulate the mitochondrial-induced apoptosis, also reside on the ER and regulate the release and uptake of ER Ca2+ by mitochondria, and hence play a pivotal role in the apoptotic cross talk between ER and mitochondria (Oakes et al. 2006; Hacki et al. 2000).

BCL2 inhibits the oligomerization of proapoptotic BAX on mitochondria and prevents mitochondrial dysfunction (Oakes et al 2006; Hacki et al 2000). ER-targeted BCL2 is shown to sequester proapoptotic activators among the BH3-only group of proteins and protect the cells from many forms of apoptosis including the BAX-promoted apoptosis (Oakes et al. 2006). Deficiency of proapoptotic BCL2 proteins BAX/BAK protects cells from ER stress-induced apoptosis, whereas expression of ER-targeted BAK in BAX/BAK double knockout cells promotes apoptosis (Wei et al. 2001). BIM, a BH3-only activator protein of BAX and BAK, inhibits anti-apoptotic BCL2 and MCL1. In normal conditions BIM is bound to dyenin motor complexes of the microtubule cytoskeleton. In response to ER stress, BIM is released from dyenin, and translocates to the ER. Apparently it is the phosphorylation of BIM by JNK that facilitates its release from dyenin motor complexes (Yoshida, 2007; Szegezdi et al. 2006). The antiapoptotic protein BCL-x_L binds to BIM and inhibits its translocation to the ER (Yoshida, 2007).

Cellular Ca^{2+} is primarily stored in ER. Since ER and mitochondria are in close proximity with each other, the release of Ca^{2+} from ER generates high calcium microdomains (~50-100 μM) at the ER-mitochondrial junctions and activates a low affinity mitochondrial Ca^{2+} uniporter resulting in mitochondrial Ca^{2+} uptake (Demaurex and Distelhorst, 2003; Rizzuto et al. 1998). Disturbances in ER–mitochondrial Ca^{2+} levels that lead to leakage of ER Ca^{2+} and uptake by mitochondria, trigger disruption of mitochondrial membrane potential, activation of a

permeability transition pore (PTP) and cytochrome *c* release, that initiates the assembly of apoptosomes and subsequent activation of the caspase cascade (Oakes et al. 2006; Demaurex and Distelhorst, 2003). Reduction in ER-Ca^{2+} load offers protection whereas increasing the ER-Ca^{2+} load sensitizes cells to ER-stress induced apoptosis (Oakes et al. 2006). The ability of the cells to transfer ER-Ca^{2+} to mitochondria defines the severity of apoptosis and agents that can promote the uptake of released ER-Ca^{2+} by mitochondria accentuate the process (Oakes et al. 2006; Demaurex and Distelhorst, 2003). Over-expression of BCL2 protects against thapsigargin induced apoptosis by due to the inhibition Ca^{2+} intake and release by SERCA (Sacroplasmic endoplasmic reticulum Ca^{2+} ATPases) pump and ryanodine receptor respectively (Pinton et al. 2001; Demaurex and Distelhorst, 2003). In contrast over-expression of pro-apoptotic BAX and BAK proteins results in Ca^{2+}mobilization from the ER to mitochondria and apoptosis (Pinton et al. 2001; Demaurex and Distelhorst, 2003). BAX/BAK-deficient mouse embryonic fibroblasts (MEFs) have lowered ER-Ca^{2+} load, and are highly resistant to Ca^{2+}–dependent apoptotic stimuli. BAP31 (B cell receptor-associated protein 31), an ER-transmembrane protein that exists in complex with cytosolic procaspase-8 is cleaved during ER stress. The cleaved pro-apoptotic p20-BAP31 can induce ER-Ca^{2+} release and apoptosis. Anti-apoptotic BCL2 and $BCLx_L$ associate with BAP31-procaspase-8 complex and inhibit their activation (Oakes et al. 2006).

CHOP down-regulates BCL2 expression by interacting with cAMP responsive element–binding protein, CREB, which induces BCL2 expression (Oyadomari and Mori, 2004; Szegezdi et al. 2006) whereas over-expression of BCL2 is shown to diminish CHOP induction (McCullough et al. 2001). JNK can also phosphorylate BCL2 and inactivate it (Szegezdi et al. 2006). Inactivated BCL2 fails to inhibit the pro-apoptotic BH3-only proteins and hence loses its control on ER Ca^{2+} fluxes (Bassik et al. 2004). Hence, CHOP mediated down regulation of BCL2 transcription and JNK mediated BCL2 inactivation through phosphorylation, eventually give an upper hand to pro-apoptotic members of the BCL2 family, sensitizing cells to apoptosis.

1.7: Objectives: From the above review of literature, it is quite evident that chronological aging is associated with decline in bulk protein synthesis. Earlier, several studies were carried on to elucidate the age associated changes in translational apparatus, however these studies were not carried in a comprehensive manner and

generally concentrated on specific individual translation factors or specific tissue in a model organism. The earlier results obtained from various studies did not implicate any particular translation factor but rather indicate the role of several important translation factors in the age associated decline in bulk protein synthesis, signifying the multifactorial etiology of translational modulation during aging and development in animal tissues.

Recent studies have established the assumption that eIF2α phosphorylation is a stress signal and plays a significant role in cellular stress response. Aging is associated with a decline in stress responsive capacity. Studies carried during the last decade have highlighted, i) the lynchpin role of endoplasmic reticulum in cellular stress response, ii) the importance of eIF2α phosphorylation and translational modulation in the ER-stress mediated adaptive signalling

With our expertise in analyzing translational modulation at the initiation level, in this present work we attempted to comprehensively analyze,

- The age-associated changes in the levels of several important translation initiation factors
- The age-associated changes in the phosphorylation status of important translational initiation factors whose functions are primarily modulated through phosphorylation using the recently available commercial antibodies that can specifically recognize phosphorylated forms of important initiation factors
- The levels of two important eIF2α kinases and the modulation of eIF2α kinase activity and eIF2α specific phosphatase activity during chronological aging
- The age-associated changes in ER-stress signalling during chronological aging.

Methodology

2.1: Chemical and Materials

2.2: Preparation of Post Mitochondrial Supernatant (PMS)

2.3: Preparation of ER enriched fraction

2.4: Preparation of Nuclear extracts

2.5: Protein estimation

2.6: *In-vitro* translation in brain extracts

2.7: *In-vitro* translation in wheat germ lysate (WGL)

2.8: Purification of GST tagged recombinant human PKR and mouse PERK

2.9: *In-vitro* phosphorylation

2.10: *In-vitro* dephosphorylation

2.11: Pull down assay

2.12: Total Polysome Profiling

2.13: Semi-quantitative RT PCR

2.14: Analysis of XBP1 mRNA splicing

2.15: Electrophoretic Mobility Shift Assay (EMSA)

2.16: Sodium dodecyl sulphate polyacrylamide gel electrphoresis (SDS-PAGE)

2.17: Wester Blotting

2.18: Densitometry

2.19: Phosphor imaging and Autoradiography

2.1: Chemicals and Materials: Wistar rats were purchased locally from National Institute of Nutrition (NIN), India. The rats were kept under standard conditions of light and temperature with commercial chow and water provided ad libitum. All the experiments were carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt of India, guidelines. Commercially obtained antibodies are as follows, Cell Signaling Technologies: eIF4E (cat # 9742), phospho-eIF4E (cat # 9741), phospho-4E-BP1 (cat # 9459), S6 (cat # 2212) and phospho-S6 (cat # 2211), and phosphor-p70S6K (cat # 9205); Santa Cruz Bioechnology Inc: JNK1 (cat # sc-1648), JNK 2 (cat # sc-7345), phospho-JNK (cat # sc-6254), eIF5 (cat # sc-282), eIF2Bε (cat # sc-9982), calnexin (cat #11397), calregulin (cat # sc-11398), GADD34 (cat # sc-794), PKR (sc-6282), actin (sc-8432) and CHOP (cat # 7351); Research Genetics: phospho-eIF2α (cat # RG0001); Calbiochem: anti GRP-78/BiP. Polyclonal ATF4 and monoclonal PERK and eIF2α antibodies were obtained as gifts. The ALP conjugated, mouse and rabbit secondary antibodies and ALP substrates, BCIP and NBT were purchased from Promega. Glutathione agarose (cat # G4315) was purchased from Sigma. Biochemicals used for the preparation of reaction mixtures, cell extracts, gel solutions and buffers were purchased from Sigma, Amersham, Promega and Calbiochem respectively. [γ–^{32}P]-ATP and [^{35}S]-methionine were obtained from BRIT and JONAKI. All the enzymes required for RT-PCR were purchased from Invtirogen and Novagen. pGEX-4T-PERK-GST plasmid was obtained as a gift from Prof. David Ron, Skirball Institute of Molecular Medicine, New York, USA. pGEX-4T-PKR-GST was as a kind gift by Bryan R.G. Williams, Lerner Research Institute, Cleveland Clinic Foundation, Ohio, USA.

2.2: Preparation of Post Mitochondrial Supernatant (PMS):

For preparation of PMS a group of 4-5 wistar rats of age, suckling (0-4 day old), young (1 month old), adult (~ 6 months old) and old (> 18 months old) were sacrificed by decapitation. The tissues were immediately dissected out, washed in cold PBS to remove residual blood, blotted dry and minced into small pieces. The minced pieces were homogenized in 1:4 volumes of buffer H (50 mM Hepes-KOH pH 7.5, 140 mM potassium acetate, 4 mM magnesium acetate, 2 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 50 mM sodium fluoride, 20 mM β-glycerophostphate, 20 mM sodium molybdate, 2 mM benzimidine, 0.32 M (only for brain) or 0.032 M (for all

other tissues) sucrose, 100 μM PMSF, 10 μg/ml of aprotinin, leupeptin and pepstatin and 0.1% triton X-100. The homogenate was spun at 11000 g for 20 min to obtain the PMS, the top ¾th supernatant was then immediately aliquoted in small volumes of 100 μl, flash freezed in liq N_2 and stored at -70^{o}C until further use. After dissecting the tissues, all the other steps were performed on ice, in a cold room.

2.3: Preparation of ER enriched fraction: For the preparation of ER enriched fractions, the PMS was prepared with buffer H supplemented with 250 mM sucrose. Later the ER enriched pellet was obtained by centrifuging the PMS at 30000 rpm for 30 min. The ER enriched pellets were dissolved in buffer containing in 8 M urea, 4% chaps and 100 mM DTT. The solubilized ER enriched protein mixture was immediately flash freezed in liq N_2 and stored at -70^{o}C until further use.

2.4: Preparation of Nuclear extracts: Nuclear pellet was obtained by spinning the tissue homogenate used for PMS preparation at 1000 rpm for 15 min. The nuclear pellet was dissolved in buffer containing 400 mM NaCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM EGTA, 5 mM β-mercaptoethanol, 1% glycerol in 30 mM Tris-HCl pH 8.0. The solubilized protein mixture was immediately flash freezed in liq N_2 and stored at -70^{o}C until further use.

2.5: Protein estimation: Protein estimation was done by bicinhoninic acid (Sigma Cat # D8284) and bradford (Sigma Cat # B6916) reagents according to manufacturer's instruction.

2.6: *In-vitro* translation in brain extracts:

The complete reaction system in a final volume of 50 μl contained 50 mM Hepes-KOH pH 7.5, 150 mM potassium acetate, 5 mM magnesium acetate, 2.5 mM DTT, 0.32 M sucrose, 1 mM ATP, 0.75 mM GTP, 20 mM phosphocreatine, 150 μg/ml creatine kinase, 50 μM amino acids (-Met), 100 μg of PMS and 5 μCi of [^{35}S]-methionine. The reaction was carried out at 30°C for 45 min. About 10 μl of reaction mixture was spotted on filter paper disc at every 15 min interval and air dried. At the end of reaction, the dried filter disc were sequentially treated in ice cold TCA for 1hr, boiling TCA for 3-4 min, 5% TCA at room temperature for 5 min, ethanol and acetone for 3 min each. After all treatments the filter discs are air dried. The dried filter discs were used for measuring the incorporated radioactivity in a liquid scintillation counter.

2.7: *In-vitro* translation in wheat germ lysate (WGL): The mRNA purified from brain and liver of young and old wistar rats was translated *in-vitro* in WGL *in-vitro* translation kit purchased from Promega (Cat # L4380) as per the manufacturer's instruction. The translated products were separated on 10% SDS-PAGE, later the gel was coomassie stained and dried. The dried gel was exposed for 1 day to phosphor imager screen (Amersham) and scanned the next day in Typhoon scanner (Amersham). 5 μCi of radiolabelled [^{35}S]-methionine was used in every 50 μl reaction to label the *in-vitro* translated products. About 5 μg of mRNA purified from rat tissues was used for each reaction. A positive control reaction was also set up using 1μg of Brome Mosaic Virus (BMV)-RNA. The translation of BMV-RNA yields four proteins of the following sizes: 110 kD, 97 kD, 35 kD and 20 kD respectively.

2.8: Purification of GST tagged recombinant human PKR and mouse PERK:

Transformation: 10 ng of plasmid DNA (PKR-GST-pGEX-4T / PERK-GST-pGEX-4T) was added to *E. coli* BL21 competent cells (100 μl) and incubated on ice for 30 min, followed by heat shock for 30-45 s at 42^{o}C and then transferred immediately on to ice. To this mixture 500 μl of LB medium (1 liter LB medium contains 10 gm NaCl, 5 gm yeast extract and 10 gm bactotryptone) containing 100 μg/ml of ampicillin was added and incubated at 37^{o}C and 150 rpm for 45 min in orbital shaker. The cell culture was spun at 4000 rpm for 2 min. The supernatant was discarded and the cell pellet resuspended in 50 μl of fresh LB medium. The cell suspension was evenly spread over LB agar plate containing 100 μg/ml of ampicillin. The plates were incubated overnight at 37^{o}C. The transformed BL 21 cells harbor a plasmid containing Amp^{R} gene that confers ampicillin resistance hence they grow normally in LB medium containing ampicillin.

Over expression and purification of PKR-GST / PERK-GST: All through the protocol the LB medium used contained 100 μg/ml of ampicillin (LB-amp). The transformed *E. coli* BL21 were inoculated in 5 ml LB-amp medium and incubated overnight at 37^{o}C and 150 rpm to get a primary culture. Secondary culture was obtained by inoculating 1 ml of primary culture into 100 ml of LB-amp medium and grown overnight at 37^{o}C and 150 rpm. A tertiary culture was prepared by inoculating 5 ml of secondary culture in 500 ml LB medium and incubating at 37^{o}C and 250 rpm for 4 hr or until the culture obtained an appropriate turbidity of ~ 0.8 at 600 nm. Then the tertiary culture was induced by addition of 2 mM IPTG and incubated for 3-4 hr at 37^{o} C. The culture was

pelleted by spinning at 6,500 rpm for 15 min at 4^{o}C. The cell pellet was resuspended in NETN buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 8.0 and 1% NP-40) with protease inhibitors (0.5 mM PMSF, 10 μg/ml pepstatin, aprotinin and leupeptin). For a 50 ml culture pellet 1.6 ml of NETN buffer was used. The cell suspension was sonicated four times at 12 amps for 20 s with an interval of 1-2 min between each burst. A properly sonicated lysate will be of straw colour. The lysate was spun at 14000 rpm for 20mins at 4^{o}C. The top 3/4th supernatant was collected and filtered through 0.45 μm filter. The filtered lysate was loaded on to glutathione agarose column preequiibrated with NETN buffer. After loading the protein, the column was washed with 10 column volumes of NETN buffer or till the OD of the washed fractions at 280 nm came down to 0.001. The PKR-GST / PERK-GST protein was eluted out with 5 Mm GSH in 20 mM Tris pH 8.0. About 10 column volumes of elution buffer was used for elution. The OD for every 500 μl fraction collected was measured at 280nm and all the high OD fractions were pooled and subjected to 4 hr dialysis in NETN buffer to eliminate excess GSH. The amount of glutathione agarose resin used for every 500 ml culture pellet was according to the binding capacity of the resin as per manufacturer's instructions. The authenticity of purified recombinant kinases was established by identifying them with their estimated molecular weights in SDS-PAGE with respect to appropriate protein molecular weight markers and also by immunoblot analysis with specific antibodies. The purified recombinant kinases were also characterized by their ability to phosphorylate eIF2α (see **Fig 2.1 and 2.2).**

2.9: *In-vitro* phosphorylation: For phosphorylation of rat tissue eIF2, a 25 μl reaction was set up by addition of 75 μg of tissue extract and 1 μg of purified recombinant eIF2α kinase PKR or PERK and 100 μM unlabelled (cold) ATP or 30 μM unlabelled ATP supplemented with 30 μCi of radiolabelled (hot) [γ–^{32}P]-ATP in phosphorylation buffer (PB) constituted of 80 mM potassium acetate, 2 mM magnesium acetate and 1 mM DTT in 20 mM Tris-HCl pH 7.8. The reaction was set up on ice and the constituents added in following order, 1: cooled nanopure water to make up the final volume to 25 μl, 2: 5 μl of 5x PB, 3: hot or cold ATP 4: 75 μg of rat tissue extract and 5: 1 μg of recombinant eIF2α kinase. The reaction is initiated by incubation at 30^{o}C for 15 min and terminated by the addition of 4x SDS-PAGE sample buffer to the reaction mix. The samples are then boiled for 3 min. The *in-vitro*

phosphorylation reactions were also supplemented with purified recombinant human eIF2α (1 μg) as and when required. The proteins in the reaction mix were separated on 10 % SDS-PAGE, transferred to nitrocellulose membrane and probed with specific antibodies. When hot ATP was used in an *in-vitro* phosphorylation reaction, the immunoblots were also visualized by phosphor imaging. In some cases *in-vitro* phoshorylation was carried out, using only the purified recombinant human-eIF2α and the recombinant PRK/PERK kinase. The inhibitory effect of rat tissue extracts on eIF2α phosphorylation was analyzed by the addition of concentrated or un-concentrated tissue extracts obtained from young and old rats to an *in-vitro* phosphorylation reaction of recombinant human-eIF2α and PKR-GST.

2.10: *In-vitro* dephosphorylation: Phosphorylation mixture containing ^{32}P labelled *in-vitro* phosphorylated recombinant human eIF2 and recombinant mouse eIF2α kinase PERK was passed through ultrafiltration columns to remove the excess radiolabel. The dephosphorylation reaction was set up in dephosphorylation buffer (20 mM Tris-HCl pH 7.4, 50 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and 800 μM unlabelled ATP) by addition of pre-phosphorylated human eIF2α and PERK-GST mixture free of extra [γ–^{32}P]-ATP to 75 μg of tissue extracts prepared with and without phosphatase inhibitors and incubated for 10 min at 30^{o}C. The dephosphorylation reaction was stopped by the addition of 4x SDS-PAGE sample buffer and then boiled for 3min. The proteins were separated on 10% SDS-PAGE, transferred to nitrocellulose membrane and probed with respective antibodies. The immunoblots were analyzed by phosphor-imaging to evaluate the loss of radioactivity upon dephosphorylation.

2.11: Pull down assay: To analyze the presence of any inhibitory interacting proteins or substances in rat tissue extracts, the purified recombinant human PKR kinase (30 μg) was incubated with 500 μg of respective tissues extracts obtained from young and old rats in PBST (Phosphate buffered saline containing 0.05% tween-20 supplemented with protease inhibitors such as 100 μM PMSF and 10 μg/ml each of leupeptin, pepstatin, and aprotinin) for 30 min at room temperature. The mixture was then added to 50 μl of glutathione agarose matrix pre-equilibrated with PBST buffer and incubated on ice for 45 min. The beads were pelleted by centrifugation at 3500 rpm for 3 min and then washed thrice with 1 ml of PBST. The GST-tagged PKR was eluted or separated out of the tissue extracts by 5 mM GSH in PBST. The eluted PKR

fraction was dialysed against PBST to remove excess GSH. The dialyzed protein was concentrated using spin columns (30 kDa cut). The concentration of the concentrated PKR-GST protein estimated and equal amounts of recombinant PKR-GST separated out of each tissue extract was used for *in-vitro* phosphorylation experiments as described above.

2.12: Total Polysome Profiling: For profiling of total polysome, freshly dissected rat tissue were immediately homogenized in 5 volumes of buffer U (200 mM Tris-HCl pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 100 μg/ml heparin, 2% polyoxyethylene 10-tridecyl ether (PTE) and 1% sodium deoxycholate) in cold room/on ice. The homogenate was briefly spun at 27000 g in a swinging bucket rotor for 5 min to remove debris. 4 ml of the supernatant was then directly layered on 1 ml 60% sucrose pad prepared in buffer B (50 mM Tris-HCl pH 8.5, 25 mM KCl and 10 mM $MgCl_2$) and centrifuged for 2 hr at 40000 rpm in an SW 41 rotor. The polysome-ribosome pellet was then resuspended in buffer U, and OD at 260nm estimated. Equal quantity (100 -200 μg) of polysome-ribosomes isolated from specific tissue of rats of various ages was diluted to 200 μl with buffer U and then layered on 15-60% sucrose gradients prepared in buffer B and centrifuged at 45000 rpm in SW 50 rotor for 1 hr. The polysome profiles were obtained by scanning the centrifuged sample at 254 nm in an ISCO (model 185) density gradient fractionator. For a linear 15-60% sucrose gradient formation, 2.3 ml of 15% sucrose solution was layered gently on 2.3 ml of 60% sucrose solution, without disturbing the interface between two solutions. The tubes were sealed with parafilm and layered horizontally for 3 hr prior to the centrifugation.

2.13: Semi-quantitative RT PCR: The total RNA were isolated from 100 mg of young and old rat brain, liver and lung using SV Total RNA isolation system (Cat # Z3100 from Promega), according to the manufacturers instruction. The concentration of RNA determined by measuring OD at 260 nm and integrity of isolated total RNA accessed by RNA formaldehyde gels. Only intact total RNA was used for the preparation of cDNA. The 20 μl reverse transcriptase reaction using 5 μg of total RNA was peformed using M-MLV reverse transcriptase kit (Invitrogen) as per the manufacturer's instruction. After obtaining the cDNA, a 25 μl PCR reaction was set up, by addition of 2.5 μl of 10X PCR buffer (Promega), 0.5 μl of RT product (cDNA), 0.5 μM primers, 6.25 mM $MgCl_2$, 200 μM dNTPs, and 2.5 units of Taq DNA

polymerase (Promega). The PCR consisted of an initial denaturation at 94^{o}C for 4 min, followed by 30 cycles containing a denaturation step at 94^{o}C for 1 min, annealing at 57^{o}C for 30 s and elongation at 72^{o}C for 1 min. Finally, an additional step cycle at 72^{o}C for 10 min was carried to end the amplification process. The primers used are as follows,

ATF4 forward primer, 5'-TCC TGA ACA GCG AAG TGT TG-3',

ATF4 reverse primer, 5'-GCC AAT TGG GTT CAC TGT CT-3',

Tubulin forward primer, 5'-ATG AGG CCA TCT ATG ACA TC-3',

Tubulin reverse primer, 5'-TCC ACA AAC TGG ATG CTG C-3',

XBP1 forward primer, 5'-AAA CAG AGT AGC AGC AGC GCA GAC TGC-3',

XBP1 reverse primer, 5' GCA TCT CTA AAA CTA GAG GCT GCT TGG TG-3'.

All the primers were custom manufactured from Integrated DNA technologies Inc. For the amplification of XBP1 cDNA, annealing was done at 60^{o}C for 30 s. The ATF4, tubulin and XBP1 amplified fragments were of 404 bp, 150 bp and 600 bp respectively. The PCR products were resolved on 2% agarose gels, stained in ethidium bromide and photographed in a Biorad UV gel doc.

2.14: Analysis of XBP1 mRNA splicing: This assay was done as described by Calfon et al. 2002 and Kumar et al. 2003. To detect, active IRE1 mediated splicing of XBP1 mRNA, the XBP1 primers used in PCR were designed to encompass nucleotides 571 to 1,114 that includes the 26 nt intron. The 600 bp fragment amplified from an unspliced XBP1 mRNA sequence contains a Pst-1 restriction site. Digestion of the of above 600 bp fragment obtained from unspliced XBP1 mRNA with Pst 1 yields two fragments of ~300 bp. In contrast, the 574 bp fragment amplified from spliced XBP1 mRNA lacks the Pst-1 restriction site and does get cleaved by Pst-1 (Calfon et al. 2002). Hence, 200 ng of the PCR amplified fragment of XBP1 was digested with 0.5 U Pst 1 for 1 hr at 37^{o}C and then separated on 2% agarose gel, stained by ethidium bromide and photographed in Biorad UV gel doc.

2.15: Electrophoretic Mobility Shift Assay (EMSA): To label 100 ng of NFκB (5'-AGT TGA GGG GAC TTT CCC AGG C-3') binding sequence specific oligonucleotide with [γ–^{32}P]-ATP, a 10 µl reaction was set up, that contains 10 units of T4 polynucleotide kinase (PNK), 2 µl of 10x PNK reaction buffer and 30 µCi of [γ–^{32}P]-ATP. The reaction was carried at 37^{o}C for 1 hr and then diluted with nanopure water to 100 µl. For electrophoretic mobility shift assay a 20 µl reaction was set up

with 1 µl of the above labelled NFκB binding sequence specific probe, 10 µg of nuclear extract obtained from respective young and old rat tissues, 1 µg of poly-dIdC (to minimise non specific binding), and 4 µl of 5x binding buffer (constituted of 50mM Tris HCl pH 8, 750 mM KCl, 2.5 mM EDTA, 0.5% (v/v) triton X 100, 62.5% (v/v) glycerol and 1 mM DTT). The binding reaction was carried at 37^{o}C for 1hr. After incubation, the samples were run for 5 hr at 100 volts and 4^{o} C through 5% native polyacrylamide gels in 90 mM boric acid, 25 mM EDTA and 90 mM Tris base. The native gel was dried and subjected to phosphor imaging in a Typhoon phosphor imager (Amersham). As a positive control a competitive reaction with excess of unlabelled probe was set up prior to the addition of labeled probe, to adjudge the specificity of the reaction.

2.16: Sodium dodecyl sulphate polyacrylamide gel electrphoresis (SDS-PAGE): Proteins were separated by a modified Laemmli method (1970). 8 ml of the 10% resolving gel mixture contained the following: 1.875 ml of 2 M Tris-HCl pH 8.8, 2.5 ml of 30:0.8 acrylamide:bis-acrylamide, 75 µl of 10% SDS, 50 µl of 10% ammonium per sulphate (APS), 6µl of TEMED and 3.75 ml of water. The 5% stacking gel mix in total volume of 2.5 ml contained: 1.875 ml of water, 0.375 ml of 2 M Tris-HCl pH 6.8, 0.325 ml of acrylamide:bis-acrylamide solution, 25 µl of 10% SDS, 50 µl of APS and 6 µl of TEMED. Protein were prepared in a sample buffer containing 0.25 M Tris-HCl pH 6.8, 10% SDS, 40% glycerol, 5% β-mercaptoethanol and 0.05% bromophenol blue. Gel electrophoresis was carried out at 120 volts with Tris-SDS-Glycine buffer (0.3% Tris-HCl, 1.5% Glycine, 0.1% SDS) until the dye front ran into the lower buffer. The proteins in the gel were visualized by coomassie staining or transferred onto nitrocellulose membrane.

2.17: Western blotting: After separation of proteins on SDS-PAGE, the proteins were transferred eletrophoretically by wet method on to nitrocellulose membrane. Transfer of proteins was carried at 25 volts/100 milli-amps overnight in cold room in standard Biorad transfer unit. For a standard western blot, the transfer buffer contained 25 mM Tris buffer and 195 mM glycine in 20% methanol, however for efficient transfer of low molecular weight proteins the methanol concentration was increased to 40% and for transfer of high molecular weight proteins the methanol percentage was reduced to 10%. After the transfer the membrane is removed and stained with ponceau S stain to check for transfer of protein onto the membrane. The

ponseau S stain is subsequently removed by washing with excess 1x TBST (10 mM Tris-HCl pH 8, 150 mM NaCl containing 0.05% Tween 20). Regions of nitrocellulose membrane, free of proteins were blocked 5% milk solution prepared in TBST for 1 hr at room temperature. After briefly rinsing the blocked membrane with TBST, the membrane is incubated with a primary antibody solution at 4^{o}C overnight, with gentle shaking. Later the membrane is washed with TBST three times for 15 min each. Then membrane is incubated with appropriate alkaline phosphatase (ALP) conjugated secondary antibody for 1 hr at room temperature followed by three washes with TBST for 15 min each. Finally the blot is developed by treating with ALP substrates NBT and BCIP (66µl and 33µl of 20 mg/ml stocks) in 10 ml of ALP buffer containing 100 mM Tris-HCl pH 9.5, 100 mM NaCl and 5 mM $MgCl_2$. The solution is removed soon after the appearance of bands and the blot washed with water, air-dried and scanned. Each immunoblot was repeated atleast three times with different batches of extracts. For the immunoblot analysis of PERK, approximately 400 µg of PMS protein extract was used, and for all others approximately 50 µg of PMS extract was used.

2.18: Densitometric analysis: Densitometric analysis of the immunoblots was performed using Biorad's quantity one software as per the protocol given in the software user manual. The relative intensities obtained from three different individuals experiments were plotted as vertical bars depicted with standard error.

2.19: Autoradiography/Phosphor-imaging: The dried gels or immunoblots containing labeled proteins were exposed to a phosphor imager screen for appropriate time depending on the radioactivity label used and the amount of radioactivity incorporated as determined by the GM-counter. The exposed screens are scanned in Typhoon phosphor imager (Amersham) according to manufacturer's instructions. Alternatively, the dried gels/immunoblots were exposed to X- ray film (Kodak) at -70^{o} C for the required time and developed by a set of photographic solutions obtained commercially as per conventional autoradiography.

Fig 2.1: Purification of mammalian recombinant PKR-GST fusion protein
Recombinant human PKR fused with GST tag was over expressed in BL-21 *E. coli* cells and purified using glutathione affinity matrix as described in methodology.
Panel A: A coomassie stained gel of the purified PKR-GST fusion protein. The fusion protein PKR-GST migrates close to 97 kDa on 10% SDS-PAGE.
Panel B: A western blot of the of the purified PKR-GST fusion protein, probed with monoclonal anti-PKR antibody.
Lanes are as follows: **Mr**, protein molecular weight standards in kilo Daltons; **1,** corresponds to 10 μl of the eluted protein PKR-GST.
Panel C: As described in methodology, *in-vitro* phosphorylation of recombinant human eIF2α with purified PRK-GST fusion protein was performed, to ascertain the functional ability of the recombinant kinase (PKR-GST) to phosphorylates its substrate eIF2α. The figure is a western blot probed with phosphospecific eIF2α antibody. Lane 1: recombinant human eIF2α alone, Lane 2: recombinant human eIF2α plus PRK-GST.

Fig 2.2: Purification of mammalian recombinant PERK-GST fusion protein
Recombinant human PERK fused with GST tag was over expressed in BL-21 *E. coli* cells and purified using glutathione affinity matrix as described in methodology.
Panel A: A coomassie stained gel of the purified PERK-GST fusion protein. The fusion protein PERK-GST migrates close to 116 kDa on 10% SDS-PAGE.
Panel B: A western blot of the of the purified PERK-GST fusion protein, probed with monoclonal anti-PERK antibody.
Lanes are as follows: **Mr**, protein molecular weight standards in kilo Daltons; **1,** corresponds to 10 μl of the eluted protein PERK-GST.
Panel C: As described in methodology, *in-vitro* phosphorylation of recombinant human eIF2α with purified PERK-GST fusion protein was performed, to ascertain the functional ability of the recombinant kinase (PERK-GST) to phosphorylates its substrate eIF2α. The figure is a western blot probed with phosphospecific eIF2α antibody. Lane 1: recombinant human eIF2α alone, Lane 2: recombinant human eIF2α plus PERK-GST.

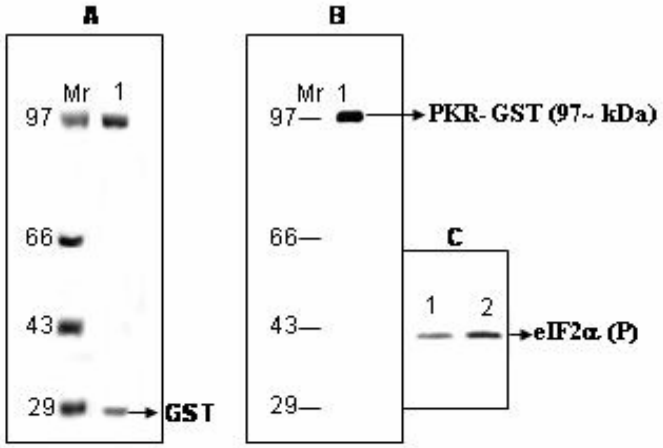
A
B
Mr 1
97
66
43
29
GST
Mr 1
97—
66—
43—
29—
PKR- GST (97~ kDa)
C
1 2
eIF2α (P)

Fig 2.1

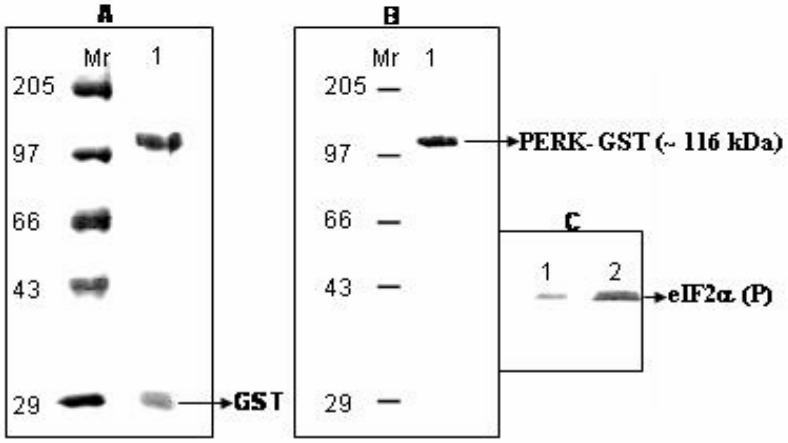
A
B
Mr 1
205
97
66
43
29
GST
Mr 1
205 —
97 —
66 —
43 —
29 —
PERK- GST (~ 116 kDa)
C
1 2
eIF2α (P)

Fig 2.2

Chapter 1

Level and phosphorylation status of various translation initiation factors during aging in different rat tissues.

3.1: Introduction: Cellular stresses profoundly inhibit translation and modulate the efficiency of several eukaryotic translational factors (Patel et al. 2002). Aging is associated with metabolic stress and detrimental alterations in cellular microenvironment that simulate a physiological stress condition. However, unlike acute stress condition, the age-associated changes occur in a progressive manner over the entire life span of an organism (Shigenaga et al. 1994; Finkel and Holbrook, 200). Inhibition of translation is one of the most rapid and adaptive stress-response that is known to conserve cellular energy and preserve the energy and aminoacid pools for important functions. Decline in bulk protein synthesis is one of the most common biochemical change observed during aging (Kimball et al. 1992; Rattan and Clark 1996; Gabius et al. 1983; Vargas and Castenada, 1984, Rattan, 1992). The implications and consequences of this detrimental change in protein synthesis are manifold, and include a decline in, the availability of enzymes, maintenance and repair systems, and in normal metabolic functioning of the cell (Rattan, 1996). Interestingly, in lower eukaryotes several studies have effectively shown that by inhibiting translation and diverting cellular energy devoted to translation towards somatic maintenance and stress repair extended the life span considerably. This observation raises a question whether general translation decline observed during aging is a cause or consequence. In this context it is highly imperative to mention that during stress rapid translational modulation of some important stress-responsive genes requires efficient translation machinery and the age-related decline might significantly compromise such rapid translational fluxes. Genomic and transcriptomic profiling for potential biomarkers of aging has been carried however since proteins are the functional components of biological system, focussing on the protein synthetic machinery and the post translational modification such as phosphorylation of important translational initiation factors during aging offers a better molecular perspective to understand mechanisms and the reason for the detrimental decline in translation during aging.

Studies on components of the protein synthetic machinery during cellular aging have revealed a decline in the efficiency of ribosomes (Rattan, 1996), a decline in the level and activity of translation initiation factors eIF2, eIF2B and elongation factors eEF1α and eEF2 (Cales et al. 1986; Kimball et al. 1992; Garcia et al. 1994; Rattan, 1991; 1992; Riis et al. 1990; Jager et al. 2002) proportional to the respective decline

in the rate of protein synthesis. However a broad study on the levels and phosphorylation status of all the major initiation factors implicated to play a regulatory role in cap-dependent and independent translation in various rat tissues has not been undertaken till date. In this work we have analyzed the levels of important translation initiation factors like eIF2, eIF4E, eIF5, eIF2B, and the small ribosomal subunit protein S6 in seven different tissues obtained from suckling (post natal 1-4 day old), young (1 month old), adult (6 months old) and old (> 18 months old) wistar rats. Post translational modification like phosphorylation is known to modulate the activity of some translation factors (Rhoads, 1999; Hershey, 1989). Hence we have also analyzed the phosphorylation status of eIF2, eIF4E, 4E-BP1, and S6 with recently available commercial antibodies that can recognize only the phosphorylated forms of these initiation factors. To evaluate the efficiency of translation initiation and elongation in rat tissues during aging, profiling of total polysomes in brain, lung, and kidney tissues of young and aged rats was also performed.

3.2: Results and Discussion:

3.2.1: Age related changes in the levels of eIF4E, phosphorylated eIF4E and 4E-BP1. The total levels and the phosphorylation status of eIF4E declined with age in brain cortex and cerebellum, lung, liver, kidney, heart and spleen tissues of wistar rats (**Fig 3.1 and 3.2**). Our results correlate with earlier reports that showed a proportional decline in general protein synthesis with age (Vargas and Castenada, 1984; Gabius et al. 1983; Kimball et al. 1992). Although the levels of eIF4E stabilized or did not show a significant decline between adult and old animals, the change observed between young animals (suckling and 1 month old) relative to their aged (adult and old) counterparts was quite significant in all tissues analyzed (**Fig 3.1 and 3.2**). Especially, the suckling rats displayed very high levels of both total and phosphorylated forms of eIF4E compared to other age groups correlating with the high amounts of protein synthesis needed for their growth and development. eIF4E, that initiates the assembly of eIF4F complex on 5'cap of mRNA by recognizing the 7-methyguanosine cap appears to be an important determinant of translation in most cell types, due to its limited availability (only 0.01 - 0.2 molecules per ribosome) compared to other factors (0.5 – 3 molecules per ribosome) (Hiremath et al. 1985; Kapp and Lorsch, 2004). It is an important factor for the regulation of protein synthesis during cell division and overexpression of eIF4E is implicated in oncogenic transformation

Fig 3.1 and 3.2: Immunoblot analysis of age-related changes in the levels of eIF4E (Fig 3.1) and phosphorylated eIF4E (P-eIF4E) (Fig 3.2) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cerb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. Rl, stands for rabbit reticulocyte lyaste used as positive control. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

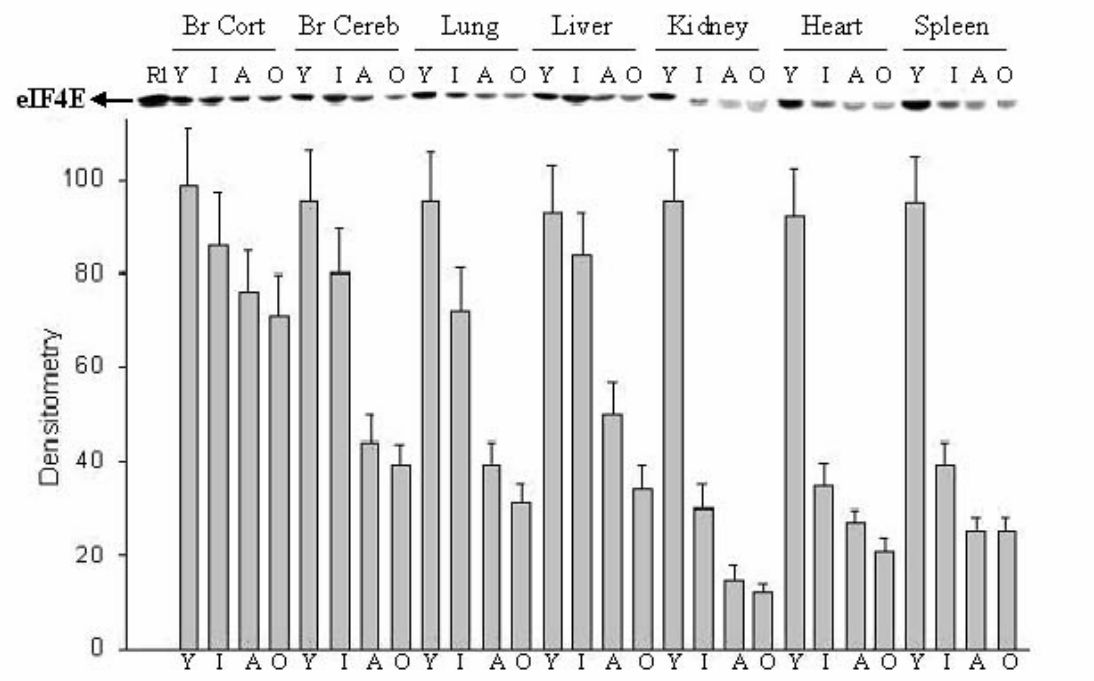
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
R1Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
eIF4E
Densitometry
0
20
40
60
80
100
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.1

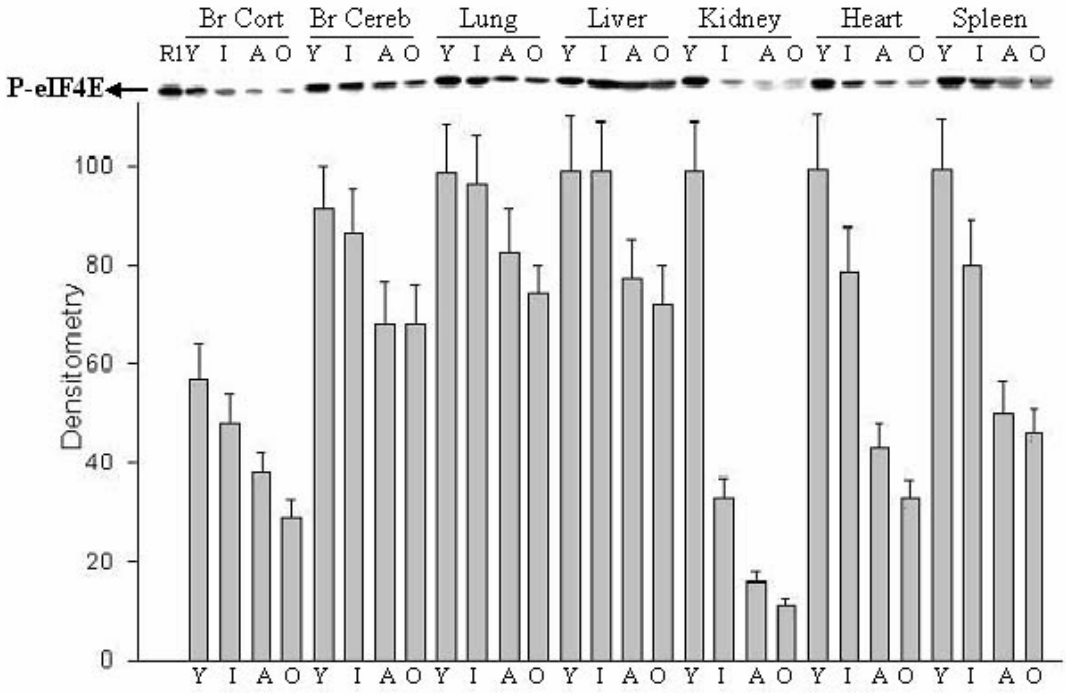
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
R1Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
P-eIF4E
Densitometry
0
20
40
60
80
100
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.2

(Miyagi et al. 1995). Overexpression of eIF4E helped to overcome the inhibitory influence of extensive secondary structure in synthetic 5' UTRs (Koromilas et al. 1992). Hence it is interesting to speculate that an active eIF4E by recruiting eIF4A might help to unwind the complex 5' UTRs present generally in growth related mRNAs (Koromilas et al. 1992). eIF4E phosphorylation at ser^{209} position by Mnk-1 generally correlates with increased translation rates (Pyronnet et al.1999). Upon mitogen and stress stimulation the MAP kinases converge on eIF4E phosphorylation via Mnk-1 activation and regulate eIF4E activity during growth and development where specific mRNAs need to be selectively translated (Raught and Gingras, 1999).

The eIF4E activity is also modulated by the inhibitory eIF4E binding proteins (4E-BP's). Among the three known isoforms of 4E-BP's, 4E-BP1 is the best characterized. 4E-BP1 and eIF4G share a common binding site on eIF4E, hence the interaction between 4E-BP1 with eIF4E blocks eIF4F complex formation by preventing the association of eIF4G (Tee and Blenis, 2005). The affinity of 4E-BP1 for eIF4E is regulated by 4E-BP1 phosphorylation. Hormones, growth factors, cytokines and mitogens, amino acid availability, and energy state of the cell have all been reported to induce phosphorylation of 4E-BPs through PI3K and mTOR kinases (Raught and Gingras, 1999; Hay and Sonenberg 2004) showing a positive correlation between 4E-BP1 phosphorylation and growth stimuli. We observed a decline in the phosphorylated levels of 4E-BP1 as a function of age in all rat tissues analyzed (**Fig 3.3**). As suckling and young rats generally display greater growth rates then their older counterparts our observations that young rat tissues displayed elevated levels of phosphorylated 4E-BP1 in contrast to their aged counterparts further substantiates the above correlation between 4E-BP1 phosphorylation and growth stimuli. The result also suggests that during aging, there is a probable decline in the stimuli which initiate the activation of kinases such as Mnk1, P13K and mTOR, that effect 4E and 4E-BP1 phosphorylation.

3.2.2: Age related alteration in the level and phosphorylation status of 40S ribosome protein S6. We have observed a general decline in the level of small ribosomal subunit protein S6 during aging in most of the rat tissues analyzed except in liver, kidney and spleen (**Fig 3.4**). Since the 40S and 60S subunits are present in stoichometric proportions, a decline in the ribosomal S6 protein indicates a decline in the total ribosomal content with age and is consistent with earlier observations that indicated a decline in translation with age (Gabius et al. 1983; Kimball et al. 1992;

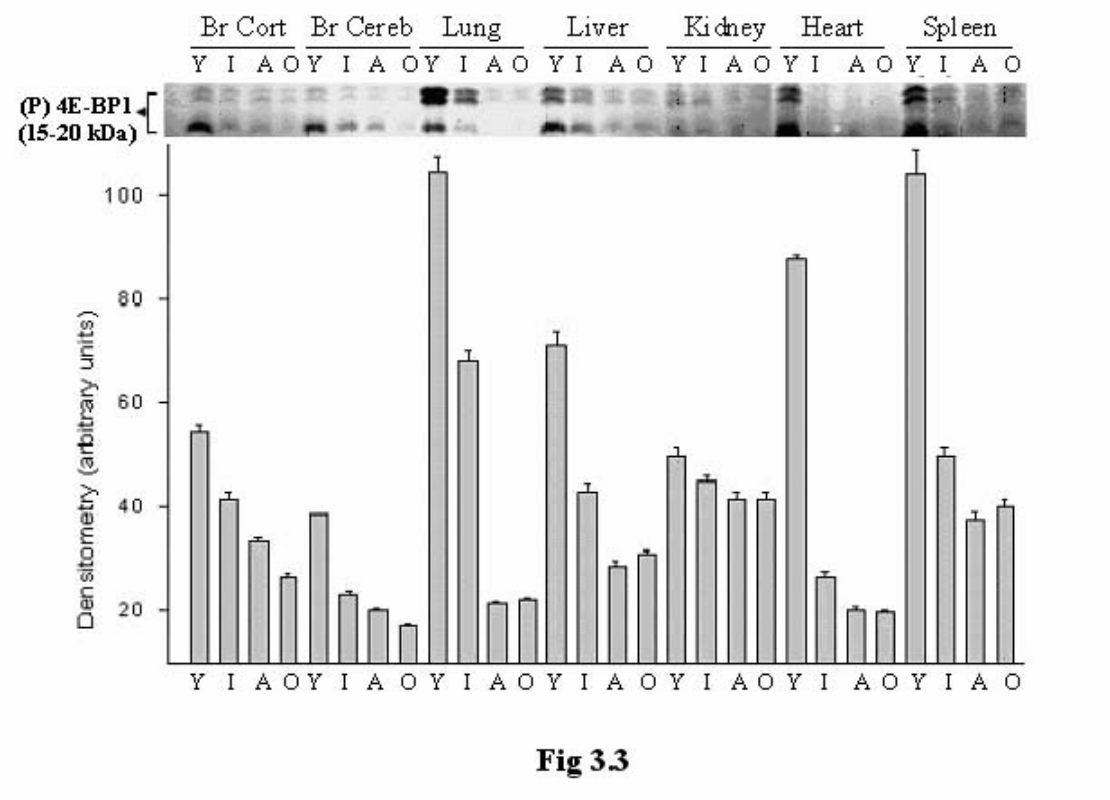

Fig 3.3

Fig 3.3: Immunoblot analysis of age-related changes in the levels of phosphorylated 4E-BP1 (P-4E-BP1) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 mg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cereb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The three bands represent different isoforms of phosphorylated 4E-BP1. The densitometric analysis with respect to the levels of β-actin is depicted as vertical bar diagram below the blot.

Vargas and Castenada, 1984). Although the mechanism is unclear, S6 phosphorylation via S6 kinase (p70S6K) has been implicated in the translational upregulation of 5' TOP mRNAs that encode mainly components of the translational apparatus, including ribosomal proteins and elongation factors (Tee and Blenis, 2005). Ribosomal protein S6, like 4E-BP1 is a common target of growth and nutrient stimuli dependent signaling pathways. The PI3K, Ras/MAPK, and mTOR kinases are known to converge on S6 kinase activation, in response to growth stimuli (Tee and Blenis, 2005). We have observed a relative decline in the phosphorylated levels of S6 protein in rats during aging in all the tissues analyzed (**Fig 3.5**). The young rats (suckling and 1 month old) displayed high levels of S6 phosphorylation compared to their aged counterparts where as the decline was minute or rather stabilized in the old rats with respect to adults (**Fig 3.5**).

3.2.3: Age related changes in the levels of eIF2, eIF2B, and the phosphorylation status of eIF2. We have observed an age related decline in the total levels of eIF2α in all the rat tissues analyzed (**Fig 3.6)**. Eukaryotic eIF2 is a heterotrimeric protein constituted of three subunits α, β and γ that occur in stoichometric proportions. eIF2 in its GTP bound form delivers the initiator Met-tRNAi to 40S ribosomal subunit and hence plays a pivotal role in the assembly of 43S preinitiation complex (Kapp and Lorsch, 2004). A decline in the level of eIF2α indicates a decline in the level of eIF2 holoprotein, suggesting that with aging, the capacity to initiate translation by forming 43S preinitiation complex declines. At the end of each round of initiation, eIF2.GTP gets released as eIF2.GDP and it has to be recycled to eIF2.GTP to take part in a fresh initiation step. eIF2B, the guanine nucleotide exchange factor catalyzes the guanine nucleotide exchange (GNE) and recycles eIF2.GDP to eIF2.GTP. The immunoblot analysis of eIFBε subunit reveals a decline in the level of eIF2B protein with aging in all the rat tissues analyzed (**Fig 3.8).** A decline in eIF2B levels indicates a decline in the capacity to recycle eIF2.GDP to eIF2.GTP with aging in rats.

Phosphorylation of eIF2α is known to inhibit the GNE activity of eIF2B, since eIF2α phosphorylated at ser^{51} residue has high affinity for eIF2B and binds it tightly acting as a competitive inhibitor of eIF2B (Ramaiah et al. 1994; Sudhakar et al. 2000; Krishnamoorthy et al. 2001). We observed a decline in the phosphorylation status of eIF2α with aging in all rat tissue analyzed (**Fig 3.7**). Although the decline was quite

Fig 3.4 and 3.5: Immunoblot analysis of age-related changes in the levels of small ribosomal protein S6 (Fig 3.4) and phosphorylated S6 (P-S6) (Fig 3.5) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cerb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. Rl, stands for rabbit reticulocyte lyaste used as positive control. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

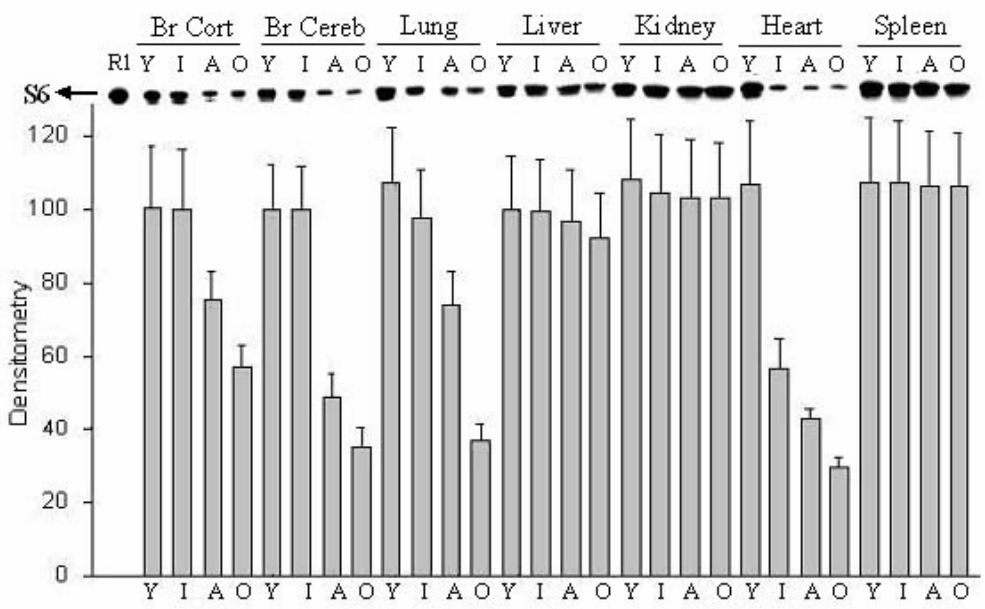
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
R1 Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
S6
Densitometry
120
100
80
60
40
20
0
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.4

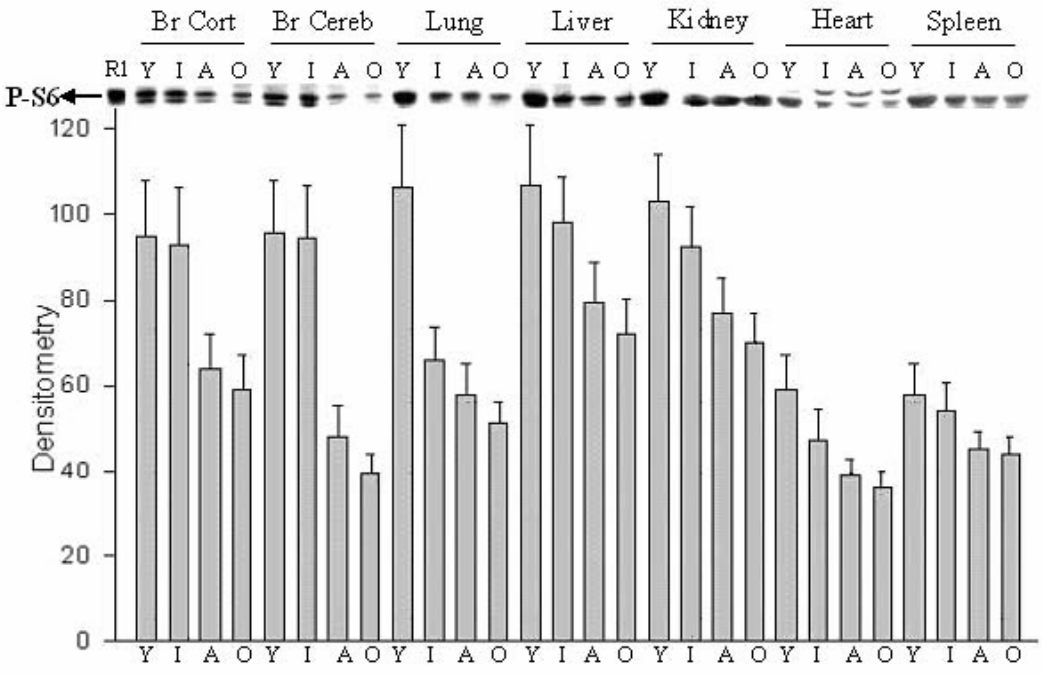
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
R1 Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
P-S6
Densitometry
120
100
80
60
40
20
0
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.5

significant between young (suckling and 1 month) and aged (adult and old) rats, the changes observed between adult and old rats were not that significant (**Fig 3.7**). eIF2α phosphorylation negatively regulates translation and this result negates the common notion that young rat display higher protein synthetic rates when compared to the older rats. However, recent studies revealed that apart from inhibiting general translation, eIF2α phosphorylation paradoxically upregulates the translation of certain gene-specific mRNAs that possess *cis*-acting features like small uORF's and IRES elements. Most of the mRNA's that encode for stress responsive proteins and growth factors possess such *cis*-acting elements (Gerlitz et al. 2002; Holcik and Sonenberg, 2005). Hence high levels of phosphorylated eIF2α observed in young tissues may be a playing an important role in normal physiological development, differentiation and stress response (Gerlitz et al. 2002; Scheuner et al 2001; Jefferson and Kimball, 2003; Brewer et al. 1999; Harding et al. 2000). In addition, the high levels of eIF2B protein present in young tissues may enable them to withstand the negative effects of eIF2α phosphorylation more efficiently than the old tissues.

3.2.4: Age related changes in eIF5 levels. eIF5, that interacts with eIF3, and with β and ε subunits of eIF2 and 2B plays a pivotal in the initiatioin step of translation. It is implicated in ternary complex recruitment, mRNA binding to ribosomes and in recognition of the AUG initiation codon. It also stimulates the intrinsic GTPase activity of eIF2 complex, an event that leads to the release of eIF2 and presumably the other initiation factors from the 48S complex, making way for 60S subunit (Preiss and Hentze, 2003). We have observed an age related decline in the levels of eIF5 in all rat tissues analyzed with the exception of brain cortex and cerebellum (**Fig 3.9**). This result also correlates with above observations implying a decline in the efficiency of translational initiation during aging.

3.2.5: Total polysome profiles of young and aged rat brain, lung and kidney tissues. From the analysis of polysome profiles in brain, lung and kidney tissues of suckling, young and aged rats, it is quite evident that the total polyribosome content declines with aging (**Fig 3.10**). The decline in the polyribosome content correlates with the observed decline in the quantity of ribosomes with aging as adjudged by ribosomal protein S6 levels (**Fig 3.4**). The decline in the high molecular weight polyribosome peak and increase in the peaks of low molecular weight polyribosomes such as monsomes and disomes suggest that the rate of initiation declines in all the

Fig 3.6 and 3.7: Immunoblot analysis of age-related changes in the levels of eIF2α (Fig 3.6) and phosphorylated eIF2α (P-eIF2α) (Fig 3.7) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cerb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. Rl, stands for rabbit reticulocyte lyaste used as positive control. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

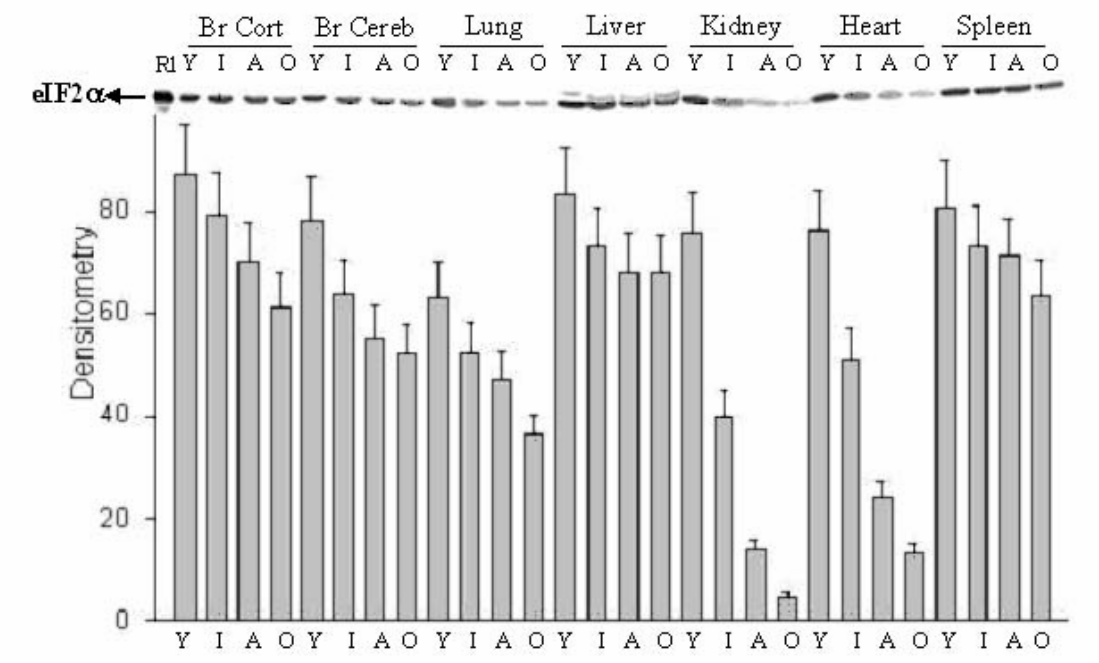
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Rt Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
eIF2α
Densitometry
0
20
40
60
80
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.6

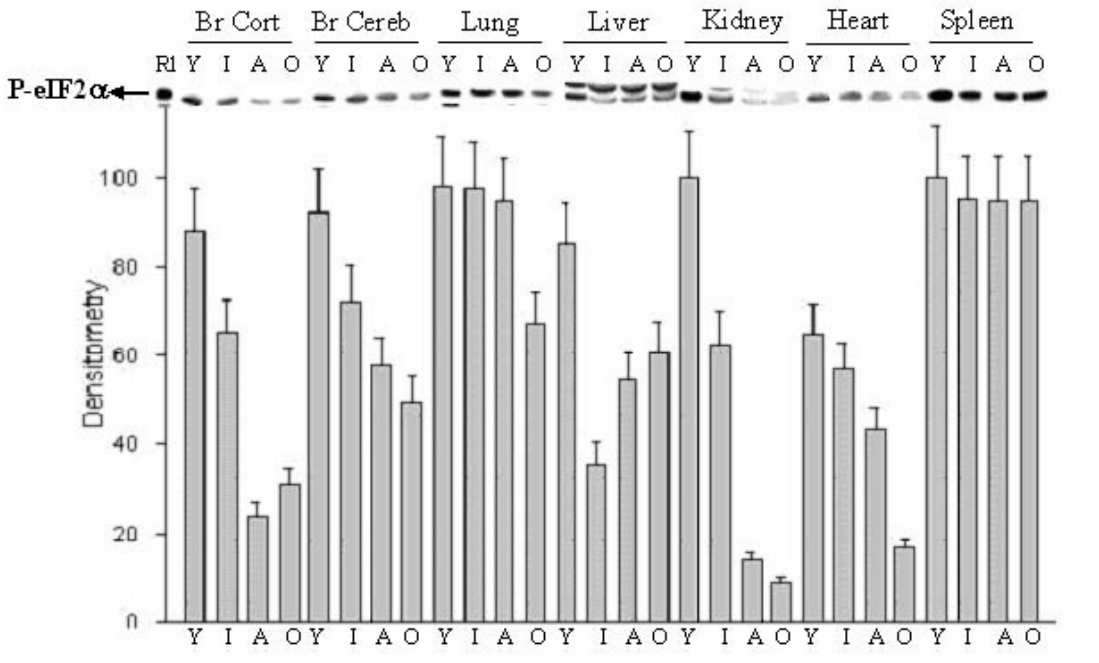
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Rt Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
P-eIF2α
Densitometry
0
20
40
60
80
100
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 3.7

Fig 3.8 and 3.9: Immunoblot analysis of age-related changes in the levels of eIF2Bε (Fig 3.8) and eIF5 (Fig 3.9) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cerb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

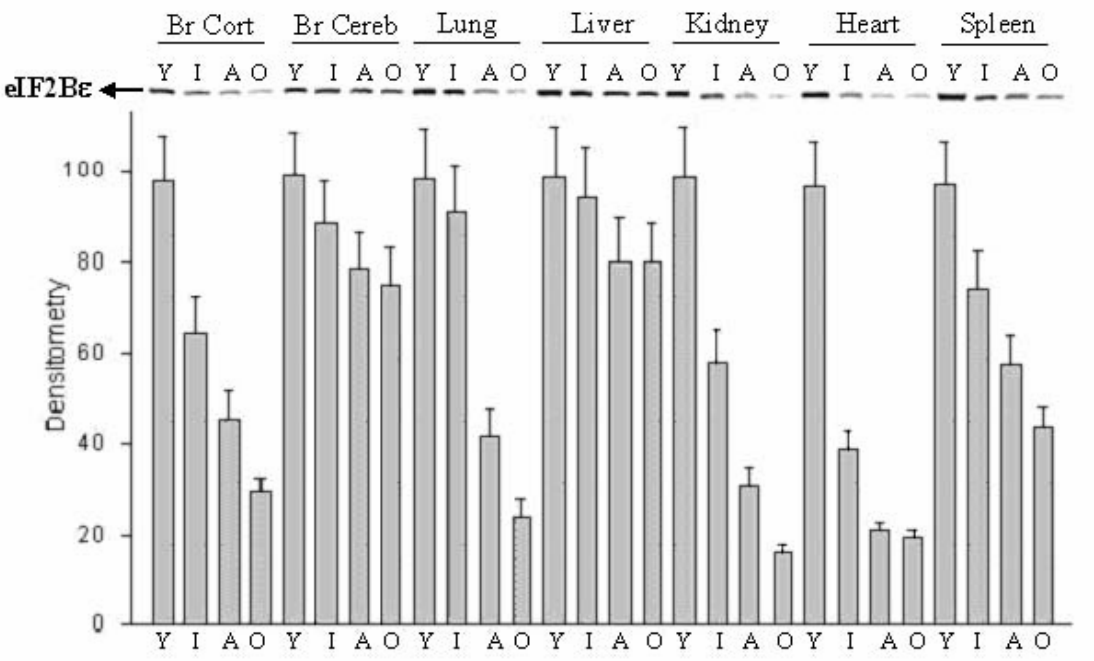
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Y I A O
eIF2Bε
Densitometry
0
20
40
60
80
100

Fig 3.8

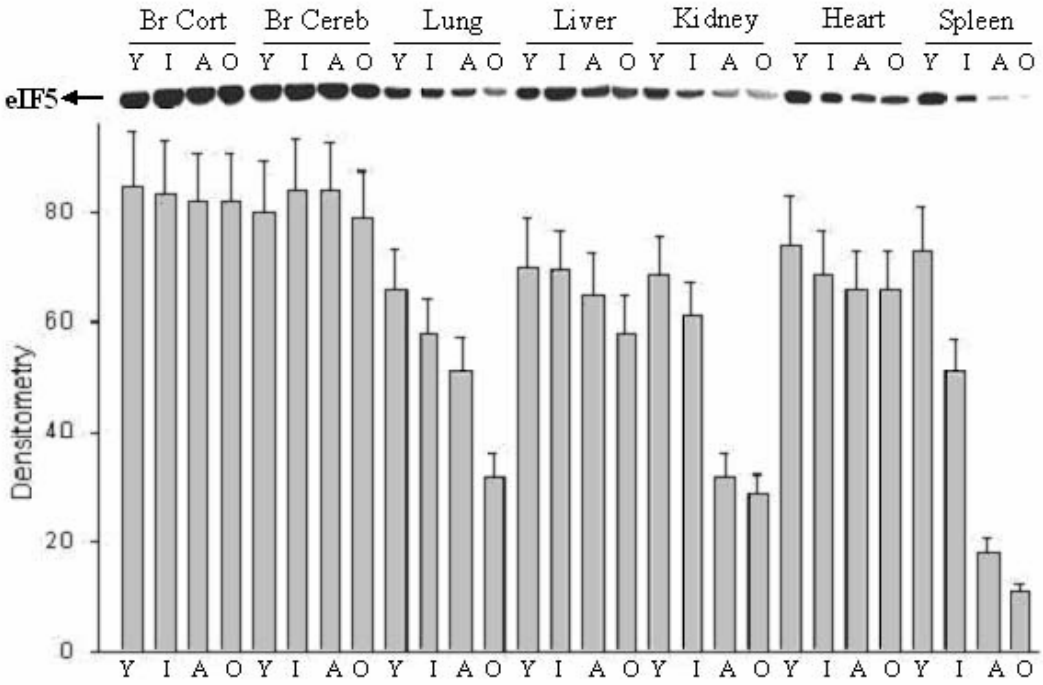
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Y I A O
eIF5
Densitometry
0
20
40
60
80

Fig 3.9

aged tissues analyzed. However, no clear evidence suggesting a decline in the elongation rates with aging is evident from the polyribosome profiles obtained in our analysis. A careful analysis of the profiles also indicates that the decline in the polyribosome content during aging is more drastic in lung and kidney than in brain tissue.

3.3: Conclusions:

Our observation reveal that young rats (suckling and 1 month old) display very high levels of eIF4E, phosphorylated eIF4E, phosphorylated 4EBP1, S6 and phosphorylated S6. S6, 4EBP1 and eIF4E function cooperatively in translational control to regulate both mammalian cell growth and proliferation in response to nutrients and growth stimuli (Fingar et al. 2002). Our observations aptly justify the above notion and correlates well with high rates of protein synthesis displayed by young rats. The age related decline in the levels of eIF2, eIF4E, eIF2B, and eIF5 relative to the levels of β-actin (**Fig 3.11**) in all the rat tissues examined suggests that with aging the efficiency of translational initiation, especially the recruitment of Met-tRNAi^{Met} and mRNA to the 40S ribosome decreases. In contrast to the high rates of translation reported in young rats, we have observed a general decline in the eIF2α phosphorylation with aging in all rat tissues examined. eIF2α phosphorylation attenuates general translation, however recent reports envisage that eIF2α phosphorylation also upregulates the translation of specific mRNAs with *cis*-acting elements in their 5' UTRs. Most of the mRNAs that code for growth factors and hormones contain such *cis*-acting elements and it is very likely that elevated levels of eIF2α phosphorylation in young rats may also mediate similar effects. We also observed high levels of eIF2B in young rats in contrast to their aged counterparts. The high levels of eIF2B may help the young rats to withstand the toxic levels of eIF2α phosphorylation more effectively that their aged counterparts. All these observations correlate positively with the age-related decline in general translation observed in many organisms (Rattan, 1992; Rattan and Clark, 1996). Our results also indicated that the levels and phosphorylation status of the initiation factors analyzed declined slightly or remained fairly constant when compared between adult and old rats. This observation correlates with a recent report that the protein synthesis in rat skeletal muscles declined up to six months and remains unchanged latter on (Kimball et al. 2004).

Fig 3.10: Analysis of total polysomes in young suckling, 1 month and aged (> 6 months) rat brain, lung and kidney tissue extracts.

Post mitochondrial supernatant (PMS) was prepared from the respective tissues using buffers designed to minimize RNAase activity. The polyribosome pellet was obtained by centrifugation of the PMS at 40000 rpm for 2 hr. The polyribosomes obtained were layered on 15-60% sucrose gradient, centrifuged for 45000 rpm for 1 hr and analyzed by ISCO gradient fractionator as described in methodology. Equal amount of the polyribosome suspension adjudged by OD @ 260 nm was layered on 15-60/% sucrose gradients for respective tissue groups.

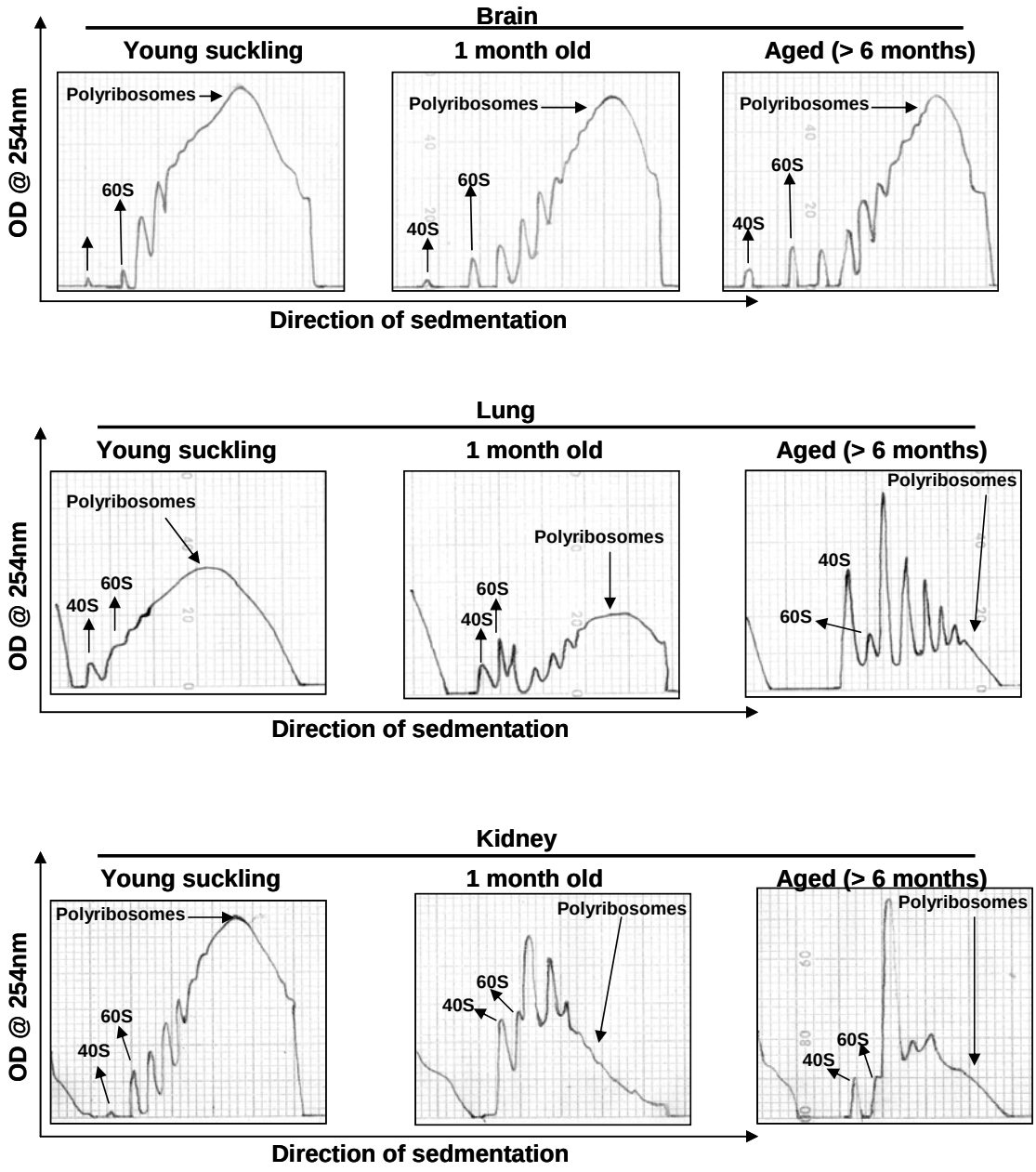
Brain
Young suckling
1 month old
Aged (> 6 months)
Polyribosomes
60S
40S
OD @ 254nm
Direction of sedmentation
Lung
Young suckling
1 month old
Aged (> 6 months)
Polyribosomes
60S
40S
OD @ 254nm
Direction of sedmentation
Kidney
Young suckling
1 month old
Aged (> 6 months)
Polyribosomes
60S
40S
OD @ 254nm
Direction of sedmentation

Fig 3.10

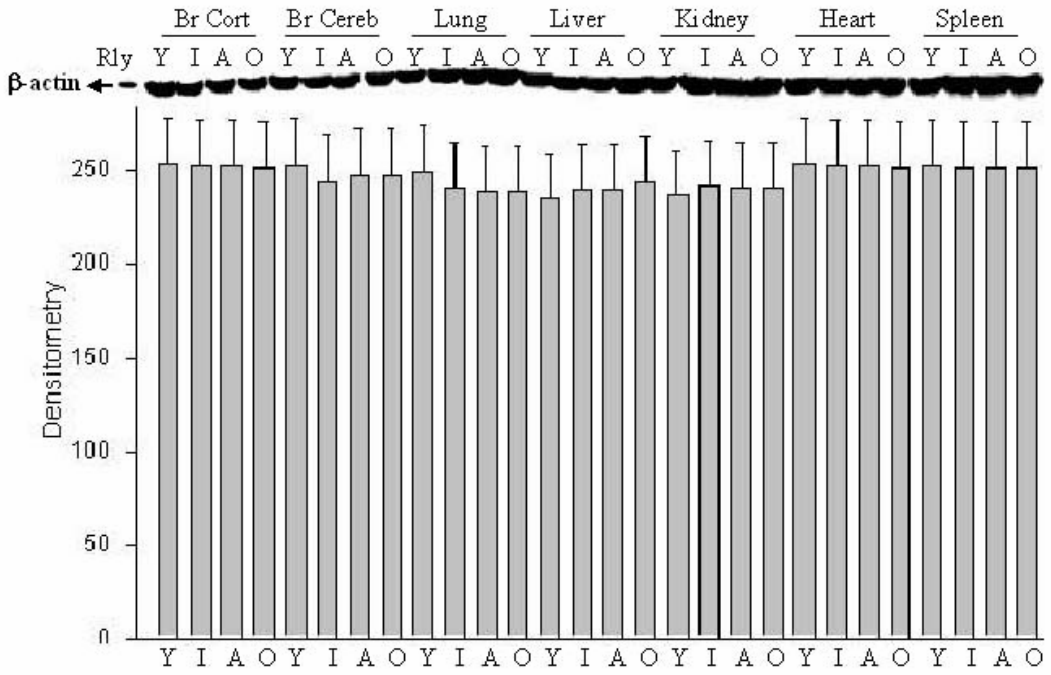

Fig 3.11

Fig 3.11: Immunnoblot analysis of β-actin was performed as described in methodology using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex (Br Cort), brain cerebellum (Br Cereb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The denstitometric analysis is depicted below the blot as vertical bar diagram.

Chapter 2

Phosphorylation and dephosphorylation of eIF2α during aging

4.1: Introduction

4.2: Results and Discussion

4.2.1: Age-associated changes in the levels of PKR and PERK

4.2.2: *In-vitro* phosphorylation of endogenous and exogenous eIF2α in rat tissue extracts

4.2.3: *In-vitro* dephosphorylation of phosphorylated human eIF2α added to rat tissue extracts

4.2.4: Levels of type 1 protein phosphatase and GADD34

4.2.5: Activity of recombinant PKR incubated and pulled down from young and old rat tissue extracts

4.2.6: Levels of ATF4, a marker of eIF2α phosphorylation

4.3: Conclusions

4.1: Introduction: eIF2α phosphorylation is stress signal, and four known eIF2α kinases, PKR, PERK, HRI and GCN2 converge upon the eIF2α phosphorylation in response to varied stress stimuli (Wek et al. 2006). Apart from inhibiting general translation, eIF2α phosphorylation also elicits a stress-responsive gene expression programme designed to alleviate cellular damage or alternatively to induce apoptosis. Through the eIF2α kinases, the cell sense, adapt and respond to various stress conditions. The eIF2α phosphorylation mediated gene expression programme is based on paradoxical translational upregulation of certain transcripts with multiple small inhibitory uORF's present in their 5'UTR in physiological conditions of low availability of ternary complex (eIF2.GTP.Met-tRNAi). Some of the transcripts upregulated in this manner are transcription factors ATF4, ATF3 (in mammalian cells) and GCN4 (in yeast) that primarily induce genes responsible for redox regulation, aminoacid metabolism, glutathione biosynthesis and anti-oxidant response (Wek et al. 2006; Hinnebusch, 1994; Harding et al. 2000; Harding et al. 2003). In addition some growth factor mRNAs with IRES elements in their 5'UTR are also efficiently translated when eIF2α is phosphorylated (Gerlitz et al, 2002; Gebauer and Hentze, 2004). This accumulating evidence suggests that eIF2α phosphorylation-mediated gene expression programme is essential for stress response, adaptation, growth, development and differentiation (Gerlitz et al. 2002; Scheuner et al 2001; Jefferson and Kimball, 2004; Harding et al. 2000). Although eIF2α phosphorylation is implicated in promoting apoptosis (Srivastava et al. 1998; Scheuner et al. 2006) it is also shown to protect cells from endoplasmic reticulum (ER) stress induced apoptosis and viral infection (Boyce et al 2005; Harding et al. 2001). The protective effects of eIF2α phosphorylation in ER stress may be mediated by the decline in client protein load on the overburdened ER and by the induction of eIF2α–specific stress responsive genes that ensure ER homeostasis. Repression of a constitutive or stress induced eIF2α–specific phosphatase activity offered cytoprotection against ER stress, viral infection and heat shock (Boyce et al. 2005; Jouse et al. 2003). Pre-emptive conditional phosphorylation of eIF2α also protected cells from lethal effects of oxidants, peroynitrite donors and ER stress (Lu et al. 2004). From the above studies it is quite evident that eIF2α phosphorylation is integral for cellular stress response. Cells that can rapidly sense stress and phosphorylate eIF2 have a better chance of survival. In addition, the eIF2α specific phosphatase activity is also essential to

maintain balance between phosphorylation and dephosphorylation and may play a pivotal role in determining the outcome of eIF2α phosphorylation mediated stress response.

The observations indicating high levels of eIF2α phosphorylation in young rat tissues relative to their older counterparts contradicts and does not correlate with the active protein synthesis displayed by young tissues. Since young tissues also displayed high levels of eIF2B, it is likely that young tissues are able to tolerate high levels of eIF2α phosphorylation. To further determine that indeed young tissues display high levels of eIF2α kinase activity that correlates to the observed eIF2α phosphorylation status, experiments with young and old rat tissue extracts, were carried out to determine, a) the levels of eIF2α kinases PKR and PERK, b) the ability of the rat tissue extracts to facilitate or inhibit the phosphorylation of exogenously supplemented human eIF2α by constitutively active recombinant eIF2α kinases, PKR and PERK, c) the ability of the rat tissue extracts to facilitate the dephosphorylation of eIF2α, d) the levels of eIF2α specific phosphatase, PP1 and the stress-induced cofactor of PP1, GADD34, and e) the levels of ATF4, a downstream target of eIF2α phosphorylation.

4.2: Results and Discussion:

4.2.1: Age-associated changes in the levels of PKR and PERK. We have observed a relative increase in the levels of two important eIF2α kinases, PKR and PERK in all the rat tissues analyzed, as a function of age (**Fig 4.1 and 4.2**). The expression of PERK was low in brain cortex and cerebellum compared to the other tissues (**Fig 4.2**). Interestingly, we have observed a decline in the levels of phosphorylated eIF2α with age in all rat tissues examined although the levels of the two eIF2α kinases increase suggesting a negative regulation of the eIF2α kinase activity in the aged rat tissues. Our observations are consistent with an earlier study in mouse revealing an enhanced expression of PKR and $p58^{IPK}$, a cellular inhibitor of PKR and PERK in aged tissues (Ladiges et al. 2000).

4.2.2: *In-vitro* phosphorylation of endogenous and exogenous eIF2α in rat tissue extracts. The above *in-vivo* observation suggests that the eIF2α kinases may be negatively regulated during aging. Hence we evaluated the activity of the kinases in the tissue extracts prepared from young and aged rats by analyzing the eIF2α phosphorylation obtained after the addition of constitutively active purified

Fig 4.1 and 4.2: Immunoblot analysis of age-related changes in the levels of PKR (Fig 4.1) and PERK (Fig 4.2) in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg (for PKR) and 400 μg (for PERK) equivalent protein of post mitochondrial supernatant of brain cortex, brain cerebellum, lung, liver, kidney, heart and spleen tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

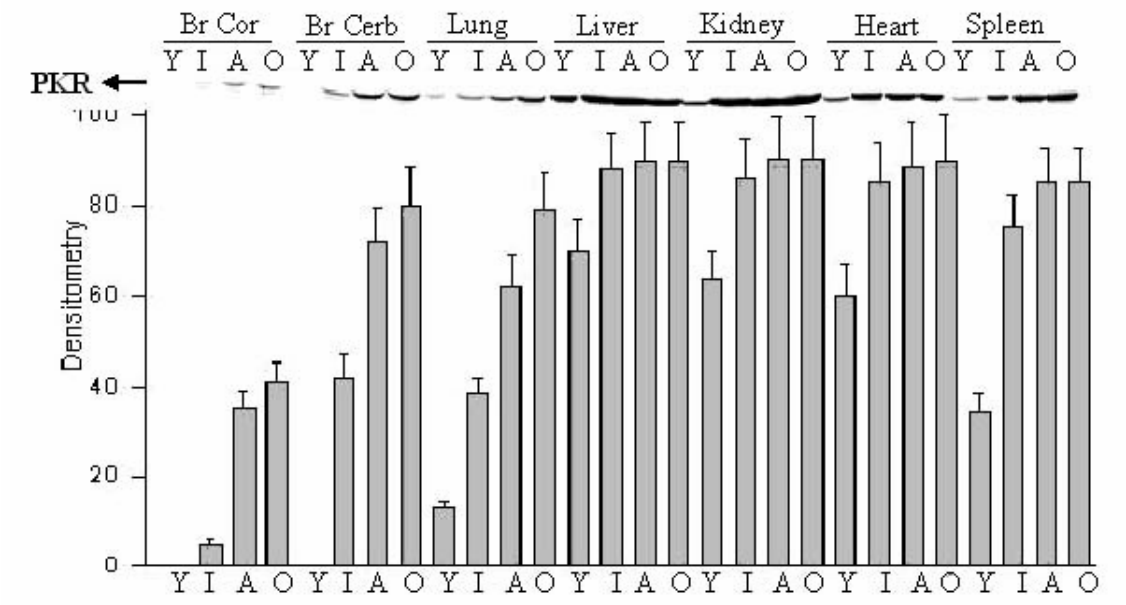
Br Cor
Br Cerb
Lung
Liver
Kidney
Heart
Spleen
Y I A O
PKR
Densitometry
0
20
40
60
80

Fig 4.1

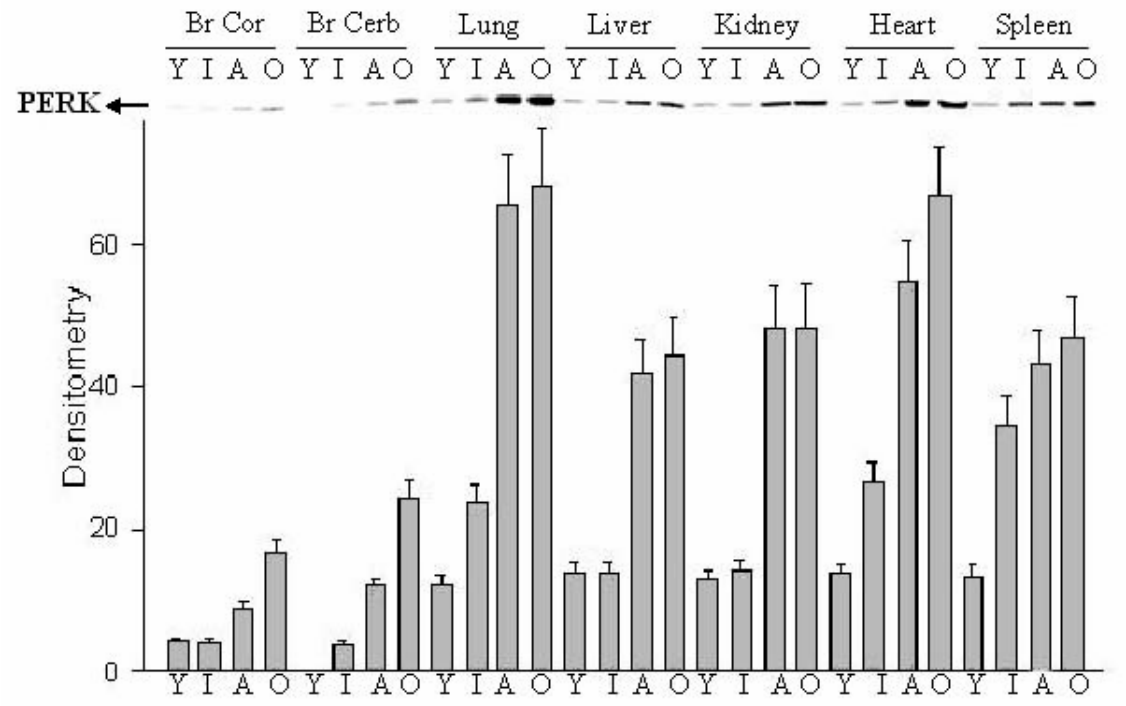
Br Cor
Br Cerb
Lung
Liver
Kidney
Heart
Spleen
Y I A O
PERK
Densitometry
0
20
40
60

Fig 4.2

recombinant human PKR or mouse PERK to rat tissue extracts. Initially, we examined the effect of recombinant human PKR in whole brain tissue extracts prepared from young and old rats on endogenous eIF2α or exogenously supplemented human eIF2α. We observed that the externally added recombinant PKR was effectively phosphorylating both endogenous rat eIF2 and exogenous human eIF2 in young brain tissue extract (**Fig 4.3,** Panel A**,** lane 1 vs 2). In contrast, the addition of the recombinant kinase to old brain extracts did not result in significant phosphorylation of exogenous and endogenous eIF2α relative to that in young brain extracts (**Fig 4.3** Panel A, lanes 1 vs 3). To further substantiate the negative effect of old brain tissue extract on recombinant PKR mediated eIF2α phosphorylatioin *in-vitro* we have added increasing concentrations (25, 50 and 75 μg) of NH_2SO_4 percipitated young and old rat brain extracts to an *in-vitro* phosphorylation reaction consisting of recombinant human PKR and human eIF2α. The results revealed that the addition of old brain tissue extract inhibited eIF2α phosphorylation in a concentration depended manner where as the addition of young brain extract did not (**Fig 4.3** Panel B lanes 1,2,3 vs 4,5,6). Even the PKR autophosphorylation was slightly inhibited by the addition of old brain extract (**Fig 4.3**, Panel B lanes 1,2,3 vs 4,5,6 in the phosphor image). Later we have also analyzed the effect of addition of recombinant mouse PERK on the phosphorylation of exogenously supplemented human eIF2α in young and old rat brain, lung, liver and kidney extracts. Surprisingly the eIF2α phosphorylation mediated by recombinant PERK was inhibited in all extracts prepared from old rat tissues (**Fig 4.4** Lanes 2 vs 4, 6 vs 8, 10 vs 12, 14 vs 16). In addition, the autophosphorylation of PERK was also less in old tissue extracts relative to young tissue extracts, as evident from the phosphorimage (**Fig 4.4** Lanes 2 vs 4, 6 vs 8, 10 vs 12, 14 vs 16). Although the exact mechanism mediating the inhibition of recombinant PERK/PKR activity in tissue extracts prepared from old rats could not be determined from our experiments we speculate that, a) the kinase activity is negatively regulated in old tissue extracts, as evident from their autophosphorylation status and eIF2α phosphorylation, and/or, b) old tissues may possess high eIF2α–specific phosphatase activity relative to young tissue leading to a rapid dephosphorylation of eIF2α in them.

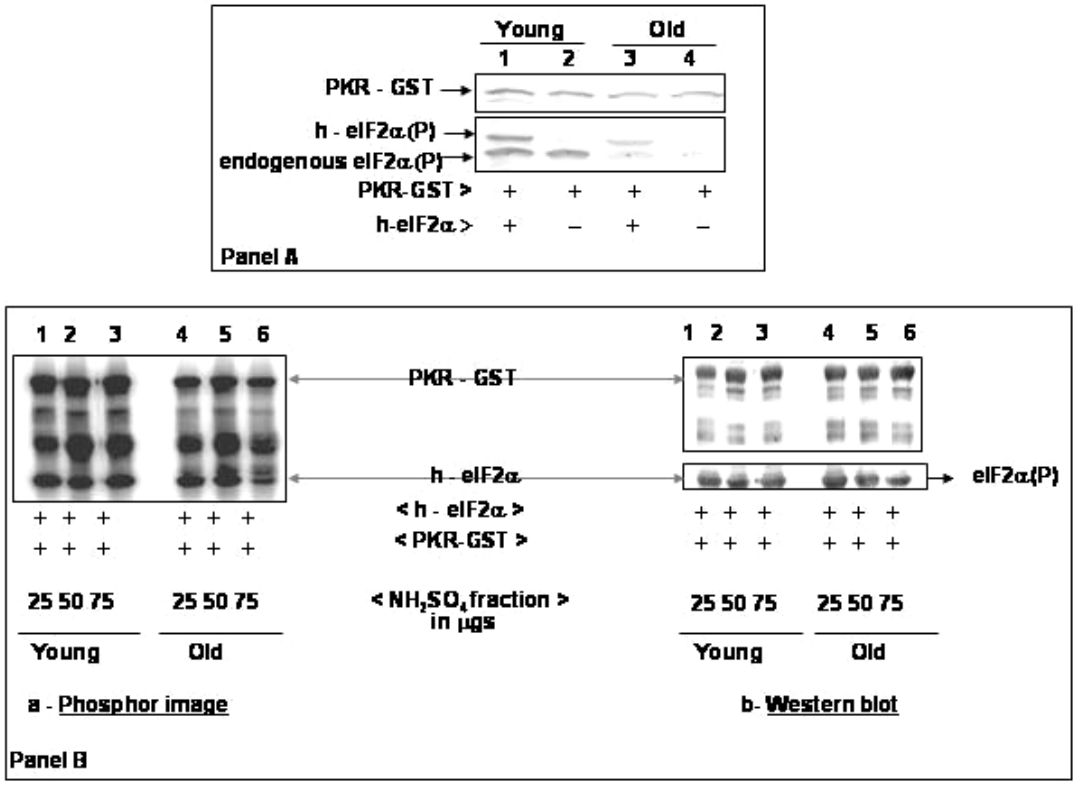

Fig. 4.3: *In-vitro* phosphorylation of eIF2α in rat brain extracts.
In-vitro phosphorylation of endogenous rat eIF2α and exogenous human eIF2α in 75 µg of post mitochondrial supernatant obtained from young and old rat brain tissue by the supplementation of recombinant human PKR as described in methodology.

Panel A: is immunoblot probed with anti-PKR and phosphospecific anti-eIF2α antibodies. Lanes are: 1, young brain extract + human eIF2α + PKR-GST, 2, young brain extract + human eIF2α 3, old brain extract + human eIF2α + PKR-GST and 4, old brain extract + human eIF2α + PKR-GST.

Panel B: is phosphorimage and immunoblot of *in-vitro* phosphorylation of human eIF2α by PKR in the presence of increasing concentrations (25, 50 and 75 µg) of NH_2SO_4 percipitated young and old rat brain extracts respectively. Lanes are: 1, 2, and 3 are PKR-GST + human eIF2α + 25,..50, and 75 µg of NH_2SO_4 percipitated young rat brain extract respectively. Lanes 3, 4, and 5 are PKR-GST + human eIF2α + 25, 50 and 75 µg of NH_2SO_4 percipitated old rat brain extract respectively.

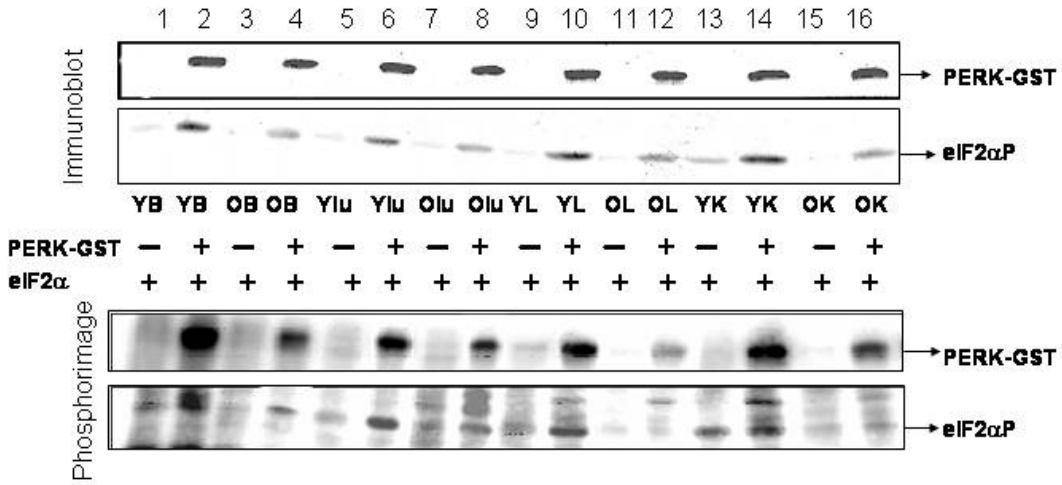

Fig 4.4: *In-vitro* phosphorylation of human eIF2α with PERK-GST in young and old rat tissue extracts.

Phosphorylation of human eIF2α was carried in 75 μg equivalent protein of post mitochondrial supernatant of brain, lung, liver, and kidney tissues obtained from young (Y) and old (O) rats, with recombinant PERK-GST as described in methodology.

Lanes are as follows:

1, 5, 9 and 13 are young brain (YB), young lung (Ylu), young liver (YL) and young kidney (YK) extract + human eIF2α respectively.

2, 6, 10 and 14 are young brain, lung, liver and kidney extract + PERK-GST + human eIF2α respectively.

3, 7, 11 and 15 are old brain (OB), old lung (Olu), old liver (OL) and old kidney (OK) extract + human eIF2α respectively.

4, 8, 12 and 16 are old brain, lung, liver and kidney extract + human eIF2α + PERK-GST respectively.

4.2.3: *In-vitro* dephosphorylation of phosphorylated human eIF2α added to rat tissue extracts. To have a better understanding of the above results we attempted to evaluate the variations in the eIF2α-specific phosphatase activity in tissue extracts of young and old rats. As detailed in material and methods a dephosphorylation reaction was set up by the addition of equal amounts of prephosphorylated recombinant human eIF2α to young and 1 month and old rat brain, lung, and liver tissue extracts prepared with and without phosphatase inhibitors. In correlation to the reduced levels of eIF2α phosphorylation (**Fig 3.7**) in aged tissues despite the presence of high levels of eIF2α kinases PERK and PKR (**Fig 4.1 and 4.2**) we observed an enhanced eIF2α–specific phosphatase activity in all the old rat tissues analyzed (**Fig 4.5** lanes 1,2 and 3,4 vs 5,6; lanes 7,8 and 9,10 vs 11,12; lanes 13,14 and 15,16 vs 17,18). Concomitant with the enhanced eIF2α specific phosphatase activity, the old rat tissues also displayed slightly higher levels of PERK-specific phosphatase activity. Dephosphorylation of eIF2α in normal physiological conditions is carried by type 1 protein phosphatase (PP1) (Babu and Ramaiah, 1996; He et al. 1997) and is specifically assisted in its function by constitutively expressed and stress-induced co-factors like CReP and GADD34 respectively (Jousse et al. 2003; Novoa et al. 2001; Marciniak et al. 2004).

The above observations point out that both the kinase and substrate are dephosphorylated more efficiently old extracts than in young extracts suggesting that the respective phosphatase activities are higher in old tissue extracts. However, we suspect that it may be apparent increase in PERK phosphatase activity then a real increase. The apparent increase may be due to the inhibition of the kinase by $p58^{IPK}$ like protein, a negative regulator of PKR and PERK which is shown induced during aging (Ladiges et al. 2000). In contrast, dephosphorylation of eIF2α, unlike PERK dephosphorylaion may be due to a real increase in the eIF2α phosphatase activity in old tissue extracts.

4.2.4: Levels of type 1 protein phosphatase and GADD34: The type 1 protein phosphatase levels do not alter with age in all the rat tissues examined (**Fig 4.6**) suggesting that the enhanced eIF2α-specific dephosphorylation activity in old rat tissues is not due to relative difference in the levels of PP1 in young and old rat tissues. GADD34, a cofactor of PP1 is shown to be induced during cellular stress conditions (Novoa et al. 2001; Marciniak et al. 2004), and it is well known that aging is associated with alteration in cellular microenvironment that resemble physiological

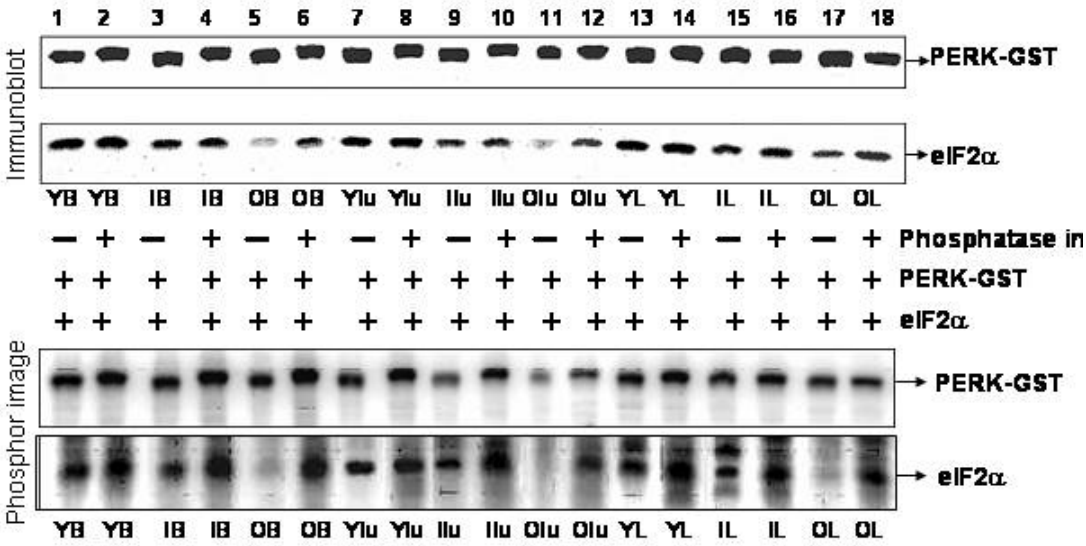

Fig 4.5: *In-vitro* dephosphorylation of pre-phosphorylated human eIF2α in rat tissue extracts.

As described in methodology, recombinant human eIF2α was phosphorylated with PERK-GST, and this phosphorylated eIF2α was used to set up dephosphorylation reaction in 75 μg of post mitochondrial supernatant prepared with and without phosphatase inhibitors, of brain, lung, and liver tissues of young suckling (Y), 1 month old (I) and old (O) rats. The figure is a phosphor image and immunoblot probed with monoclonal PERK and phosphospecific anti-eIF2α antibodies respectively.

Lanes are as follows:

1, 7 and 13 are young suckling rat brain (YB), lung (Ylu) and liver (YL) extracts without phosphatase inhibitors + human eIF2α and PERK-GST.

2, 8 and 14 are young suckling rat brain (YB), lung (Ylu) and liver (YL) extracts with phosphatase inhibitors + human eIF2α and PERK-GST.

3, 9 and 15 are 1 month old rat brain (IB), lung (Ilu) and liver (IL) extracts without phosphatase inhibitors + human eIF2α and PERK-GST.

4, 10 and 16 are 1 month old rat brain (IB), lung (Ilu) and liver (IL) extracts with phosphatase inhibitors + human eIF2α and PERK-GST.

5, 11, and 17 are old rat brain (OB), lung (Olu) and liver (OL) extracts without phosphatase inhibitors + human eIF2α and PERK-GST.

6, 12, and 18 are old rat brain (OB), lung (Olu) and liver (OL) extracts with phosphatase inhibitors + human eIF2α and PERK-GST.

Fig 4.6: Immunoblot analysis of age-related changes in the levels of PP1 in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex, brain cerebellum, lung, liver, kidney, heart and spleen tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. Rly, stands for reticulocyte lysate used as a positive control in the immunoblot. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

Fig 4.7: Immunoblot analysis of age related changes in the levels of GADD34 in various rat tissues.

Immunoblot analysis was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain, lung, liver, kidney, heart and spleen tissues obtained from young suckling (Y), 1 month old (I) and old (O) wistar rats. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot. β-actin immunoblot is shown in lower part of the figure.

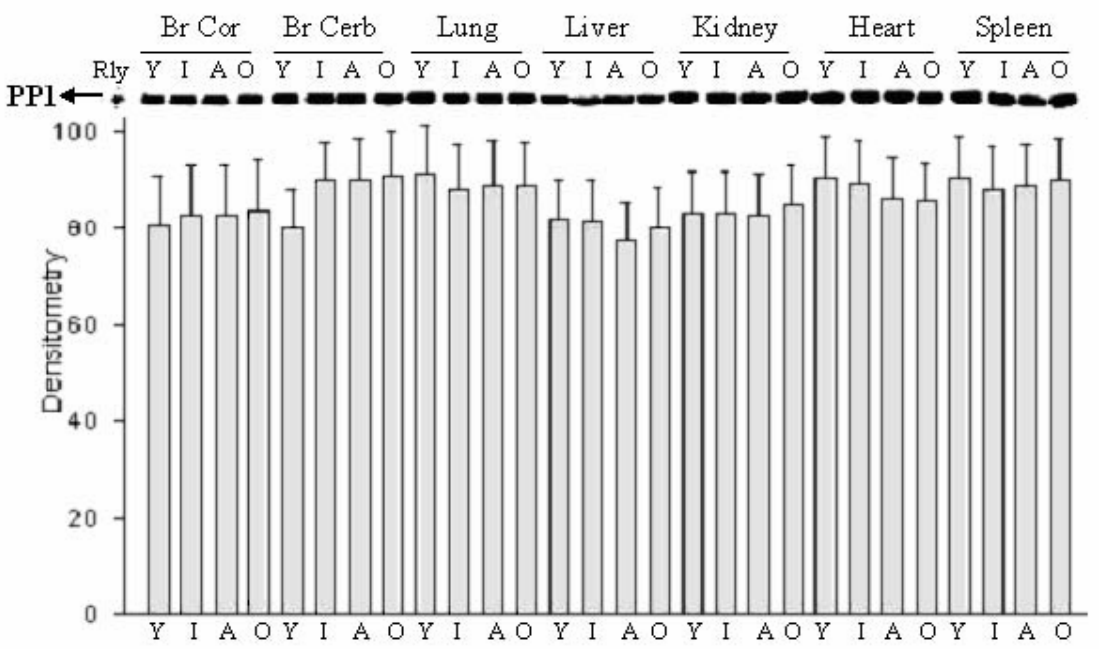
Br Cor
Br Cerb
Lung
Liver
Kidney
Heart
Spleen
Rly Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O
PP1
Densitometry
100
80
60
40
20
0
Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O Y I A O

Fig 4.6

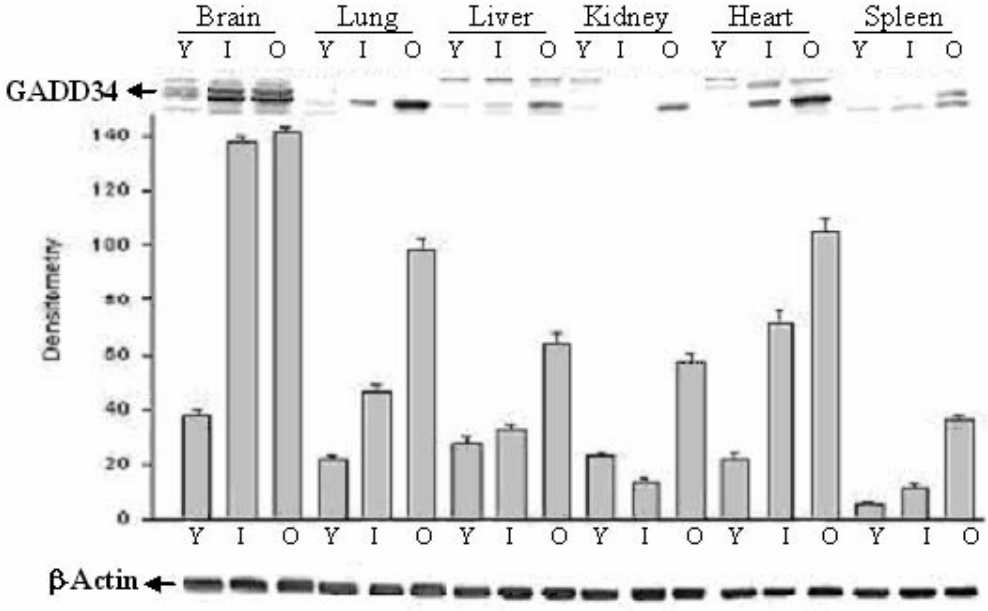
Brain
Lung
Liver
Kidney
Heart
Spleen
Y I O Y I O Y I O Y I O Y I O Y I O
GADD34
Densitometry
140
120
100
80
60
40
20
0
Y I O Y I O Y I O Y I O Y I O Y I O
β-Actin

Fig 4.7

stress stimuli. Hence, we analyzed the age-related changes in the levels of GADD34. Interestingly the levels of GADD34 are also elevated in all the aged rat tissues analyzed (**Fig 4.7**). The elevated levels of GADD34 in old rat tissues justify the enhanced eIFα-specific phosphatase activity observed in old rat tissues.

4.2.5: Activity of recombinant PKR incubated and pulled down from young and old rat tissue extracts. Since the eIF2α kinase activity was negatively regulated in old tissue extracts, we wanted to evaluate if the old tissue extracts possess any substance(s) that negatively regulate eIF2α kinases. For this an in-vitro phosphorylation reaction was set up using a recombinant GST-tagged PKR that was incubated with the brain, lung and liver tissue extracts obtained from young and old rats and then sperated out of the tissue extracts using glutathione affinity matrix. The kinase used after prior incubation in old tissue extracts did not effectively phosphorylate eIF2α and displayed less autophosphorylation than the PKR incubated in young tissue extracts (**Fig 4.8** lanes 2 vs 3, 4 vs 5 and 6 vs 7). From this result it is evident that old tissues possess properties that negatively regulate the PKR activity. However right now we cannot comment if this inhibition is specifically directed only towards PKR or in general against all eIF2α kinases.

4.2.6: Levels of ATF4, a marker of eIF2α phosphorylation. It is well known that eIF2α phosphorylation mediates the translational up-regulation of certain specific transcripts with cis-acting elements in their 5'UTR. ATF4, a bZIP transcription factor is one such transcript translationally upregulated during condition of eIF2α phosphorylation (Harding et al. 2000; 2003). Hence, ATF4 can be used as a marker for eIF2α phosphorylation. As speculated, the levels of ATF4 matched to the levels of eIF2α phosphorylation (**Fig 4.9 and Fig 3.7**). The young rat tissues displayed high levels of ATF4 relative to their aged counterparts **(Fig 4.9)**, even though the levels of ATF4 mRNA analyzed by semi-quantitative RT-PCR in brain, lung and liver tissues did not reveal any alterations with age (**Fig 4.10**). This observation correlates positively with, a) the levels of eIF2α phosphorylation observed in young tissues, b) the negative regulation of eIF2α kinases PKR and PERK observed in old tissues, and c) high levels of eIF2α specific phosphatase activity displayed by old tissues.

4.3: Conclusions: Our observations reveal that the eIF2α kinases are negatively regulated with aging is substantiated by the high levels of two eIF2α kinases, PKR and PERK in aged rat tissues albeit low levels of eIF2α phosphorylation in them. The

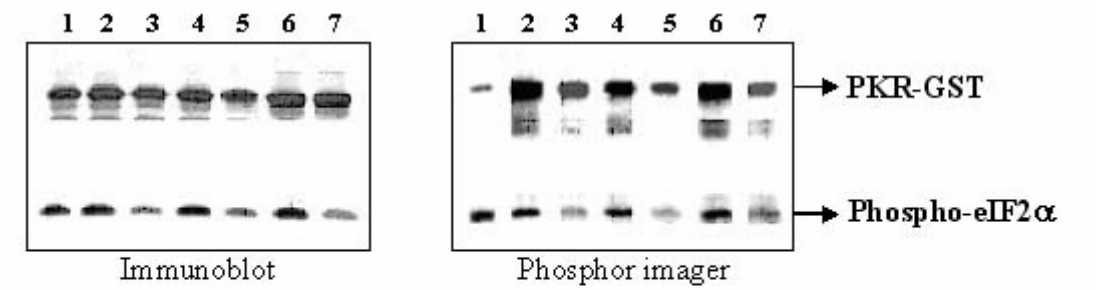

Fig 4.8

Fig 4.8: *In-vitro* phosphorylation of recombinant human eIF2α with recombinant PKR pulled out after pre-treatment in respective young and old rat tissues.

Prior to *in-vitro* phosphorylation PKR was incubated in respective young and old rat brain, lung and liver tissue extracts and then pulled out of the tissue extracts using an affinity matrix. Equal amounts of the recombinant PKR incubated and pulled out from various tissue extracts was then used to phosphorylate recombinant human eIF2α as described in methodology.
Lanes are as follows:
1: a control *in-vitro* phosphorylation reaction mix with human PKR-GST and eIF2α.
2 and 3: *in-vitro* phosphorylation with PKR-GST incubated and pulled out of young and old rat brain (YB and OB) tissue extracts respectively.
4 and 5: *in-vitro* phosphorylation with PKR-GST incubated and pulled out of young and old rat lung (Ylu and Olu) tissue extracts respectively.
6 and 7: *in-vitro* phosphorylation with PKR-GST incubated and pulled out of young and old rat liver (YL and OL) tissue extracts respectively.

Fig 4.9: Immunoblot analysis of age-related changes in the levels of ATF4 in various rat tissues.

Immunoblot analysis for ATF4 was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex, brain cerebellum, lung, liver, kidney, heart and spleen tissues of young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

Fig 4.10: Semi-quantitative RT-PCR analysis of ATF4.

The age related changes in the ATF4 mRNA levels, in brain (B), lung (lu) and liver (L) tissues of young (Y) and aged (O) rats was evaluated by semi-quantitative RT-PCR as described in methodology. Tubulin amplification was done simultaneously to show that equal amount of cDNA from each tissue was used for the RT-PCR reaction. Lanes are as follows: M: 1 kb DNA ladder, YB, Ylu and YL are young brain, lung, and liver respectively. OB, Olu and OL are old brain, lung, and liver respectively.

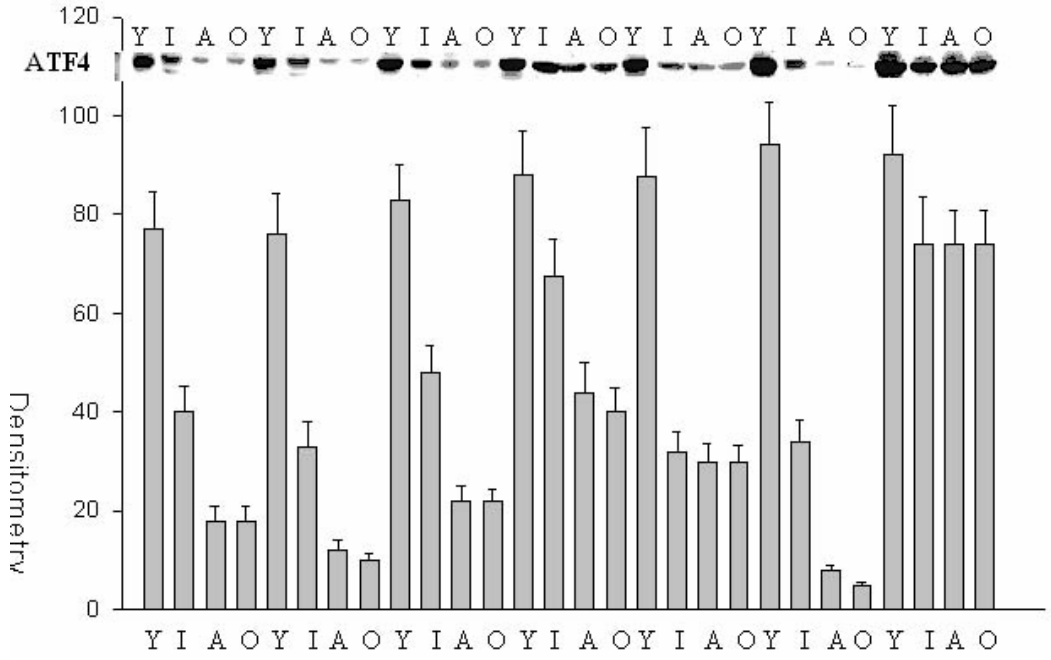

Fig 4.9

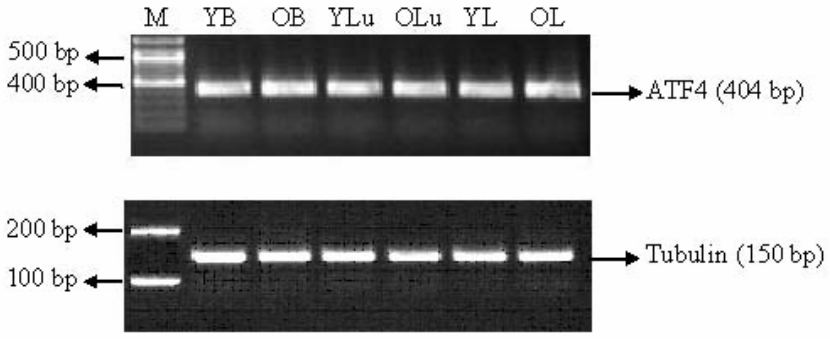

Fig 4.10

inverse correlation between the levels of eIF2α phosphorylation and the kinases in aged rat tissues is further justified by the high amounts of eIF2α specific phosphatase activity displayed by aged rat tissues. However analysis of type 1 protein phosphatase (PP1) that is known to specifically dephosphorylate eIF2α and its cofactor GADD34, indicates that the basal levels of PP1 does alter significantly but the levels of its cofactor, GADD34 increase with age. PERK activity is implicated in ER stress response (Schroder and Kaufman, 2005) while PKR activity is implicated in cytosolic stress responses (Wek et al. 2006). In addition, a recent study suggests that PKR activation also occurs during ER stress (Lee et al. 2007). The reduced activity of PKR and PERK kinases in aged tissues despite their high levels suggest that in aged tissues the antiviral, oxidative, and ER–stress responses are highly compromised. This is further substantiated by the levels of ATF4 in young rat tissues that correlates with eIF2α phosphorylation although the ATF4 mRNA levels do not alter significantly with age.

Chapter 3

Endoplasmic Reticulum (ER) stress response and aging.

5.1: **Introduction**: An organism's survival is dependent on the function of maintenance and repair capacities. Efficient DNA repair, anti-oxidant defence, and stress response capacity correlate positively with life span of the species, whereas rate of damage accumulation such as mutations, epimutations, macromolecular oxidation, and aggregation correlate negatively with longevity. However it is still unclear whether the failure of maintenance and repair systems manifests into aging or is it the response to the damage that leads to the phenotype of aging (Sierra, 2006). Several studies revealed an age associated induction of stress-responsive genes linked to immune, inflammatory and heat shock response (Prolla, 2002; Weindruch et al. 2001). In contrast genes responsible for metabolic and biosynthetic processes are down regulated as a function of age (Edwards et al. 2004; Weindruch et al. 2001).

Studies during the last decade have linked endoplasmic reticulum (ER) to cellular stress response and adaptation. The ER is devoted to synthesis, folding, processing and trafficking of secretory/membrane proteins. The highly oxidative and calcium rich ER micro-environment facilitates the functioning of a plethora of ER resident chaperones, foldases and carbohydrate processing enzymes that monitor and catalyze the protein folding process (Schroder and Kaufman, 2005). Several physiological disturbances like nutrient deficiency, limited availability of glucose and ATP, deregulated calcium homeostasis, redox imbalance, oxidative stress, expression of mutant proteins, and viral infection, etc, can perturb the ER function and lead to accumulation of unfolded protein in the ER. In order to restore homeostasis, the ER elicits a coordinated and intricate adaptive signalling cascade called the unfolded protein response (UPR) (Schroder and Kaufman, 2005). Since the ER function is dependent on several physiological parameters, the ER is highly sensitive to a cell's physiological and environment perturbations. This feature of the ER consolidates its lynchpin role in cellular stress sensing and response capabilities.

Aging is implicated in decline in the generation of ATP (Beckman and Ames 1998; Miyoshi et al 2006; Shigenaga et al. 1994), deregulation of calcium homeostasis (Ferrington et al. 1998), increased generation of ROS (Stachowiak et al. 1998; Sohal and Weindruch 1996), reduced antioxidant response (Holbrook and Ikeyama 2002, Yoon et al. 2002), increased oxidative stress and accumulation of oxidative damage to macromolecules (Stadman, 2006; van der Vlies, 2003), and reduced proteosomal activity (Rosenberger, 1991; Schwartz and Ciechanover 1999; Rattan, 1996). Taken as a whole, the age-associated decline in the cell function coupled with metabolic

stress and alteration in the cellular micro-environment may eventually trigger ER stress. Since the age-associated detrimental alterations progress and accumulate over the entire life span of an organism, it is highly probable that aged cells are under persistent ER stress. In addition ours as well as others observations suggest an age-associated decline in the efficiency of translation initiation factors (Kimball et al. 1992; Rattan and Clark 1996) which may slacken the rapid modulation of translation required for the translational upregulation of certain stress-responsive genes during stress conditions such as ER stress. There are models which suggest that ER stress may be a link between oxidative damage of proteins and the aging process (Ermak & Davies, 2002; van der Vlies et al. 2003). In *C. elegans*, ER stress is shown to determine the life span of the organisms (Viswanathan et al. 2005). Also, there is direct evidence that a proapoptotic protein GADD153 induced during ER stress is elevated in the aged liver and sensitizes aged cells to oxidative stress (Ikeyama et al. 2003; Li and Holbrook, 2004).

The ER via the UPR links the physiological stress prevalent in the cell to cytoplasm and nucleus. Three ER transmembrane proteins, IRE-1 (inositol-requiring and ER to nucleus signalling), ATF6 (activated transcription factor) and PERK, that serve as ER-stress transducers, facilitate the transduction of ER-stress signal to the cytoplasm and nucleus (Tirasophon et al. 1998; Haze et al. 1999; Shi et al. 1998; Harding et al.1999). In normal conditions, the ER-resident chaperone BiP is bound to the luminal domains of these ER-stress transducers, during ER-stress BiP gets released from them leading to the consequent activation of the ER stress transducer (Hendershot, 2004; Bertolotti, 2000). On one hand, UPR stimulates the expression of ER resident molecular chaperones and foldases to promote protein folding in the ER (Kozutsumi et al. 1998). On the other hand, UPR decreases the unfolded protein burden in the ER by stimulating ER associated degradation of slowly folding or folding incompetent proteins (Schroder and Kaufman, 2006), attenuation of the influx of nascent, unfolded polypeptide chains by inhibiting general translation (Harding et al. 1999; Shi et al. 2000), and inhibiting the transcription of genes encoding secretory proteins (Pakula et al. 2003). Although the UPR is primarily adaptive, in higher eukaryotes it can also trigger apoptosis to eliminate unhealthy cells if the ER-stress is incessant and irreparable (Schroder and Kaufman, 2006; Szegezdi et al. 2006).

In this study, we attempted to analyse the age-associated alterations in the levels of UPR markers and proapoptotic proteins induced through it to get an insight into the

ER stress response during aging. Most of the previous studies were carried out *in-vitro* in cell cultures, whereas we have used different rat tissues during chronological aging.

5.2: Results and Discussion:

5.2.1: Age-related changes in the levels of BiP/GRP78, and the lectin chaperones calnexin and calreticulin in rat tissues: Our observations reveal an age-dependent decline in the levels of ER resident chaperones BiP/GRP78 and calnexin in all the rat tissues analyzed (**Fig 5.1 and 5.2**). Interestingly, there was no significant age-dependent change in the level of calreticulin in the various rat tissues analyzed (**Fig 5.3**). Earlier studies have also reported a decline in the levels of BiP in aged mouse liver (Rabek et al. 2003) and in the levels of calnexin in *in-vitro* aged human fibroblast (Choi and Kim, 2004). Aging was also shown to increase the cumulative oxidative damage to the ER-resident proteins calreticulin, PDI (protein disulphide isomerase) and BiP in aged mouse liver (Rabek et al. 2003). In renal epithelial cells, ER-stress is shown to upregulate ER chaperones BiP/GRP78 and GRP94. Attenuating BiP expression through RNA interference rendered the cells sensitive to ER stressors and cells over expressing calreticulin resist oxidative stress, calcium disturbances and cell death (Liu et al. 1997). BiP's release is a prerequisite for the activation of the ER stress transducers and over expression of BiP suppresses the ER-stress response (Hendershot, 2004; Bertolotti et al. 2000). These findings suggest that BiP, an ER stress sensor critically regulates ER-stress response and serves as marker for ER stress response. The high levels of BiP and calnexin in young rats (**Fig 5.1 and 5.2**) suggest that young tissues experience ER stress and the adaptive ER stress response signalling pathways operate efficiently in them. Although it is well known that the aged cells experience alterations in cellular environment that lead to ER dysfunction, the observed decline in the levels of BiP and calnexin in aged rat tissues suggests that the ER-stress responsive adaptive signalling is compromised in them. The young rat tissues may experience ER-stress probably due to the high synthetic rates and nutrient requirements needed to match their rapid growth rates (Scheuner et al. 2001). Interestingly, the efficient ER stress response mediated adaptive signalling in young rats may help them sustain over the physiological stress experienced during their rapid growth and development.

Fig 5.1 and 5.2: Immunoblot analysis of age-related changes in the levels of BiP/GRP78 and Calexin in various rat tissues.

Immunoblot analysis for BiP was done as described in methodology in 50 μg of ER enriched fractions of brain (B), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from suckling (Y), 1 month old (I), and old (O) wistar rats. The densitometric analysis for each individual lane is depicted as a vertical bar diagram below the blot.

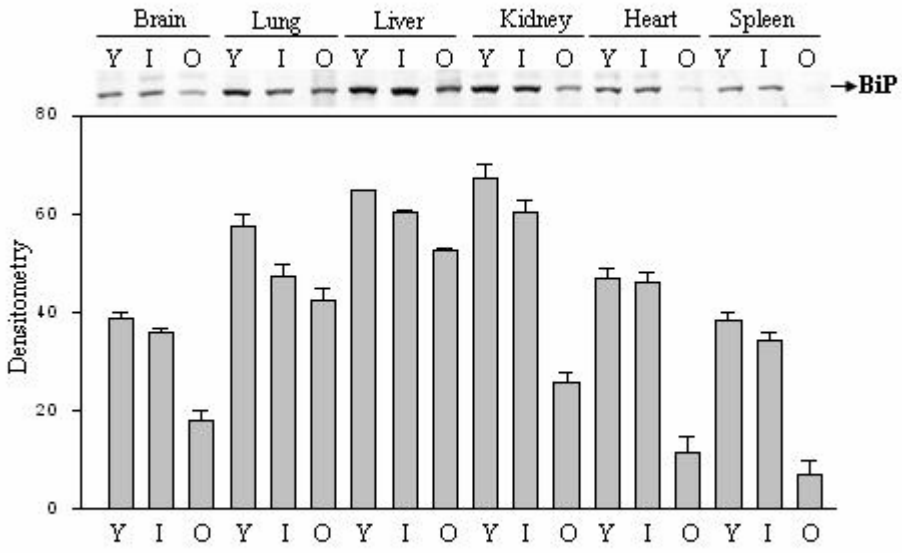
Brain
Lung
Liver
Kidney
Heart
Spleen
Y I O Y I O Y I O Y I O Y I O Y I O
BiP
Densitometry
80
60
40
20
0
Y I O Y I O Y I O Y I O Y I O Y I O

Fig 5.1

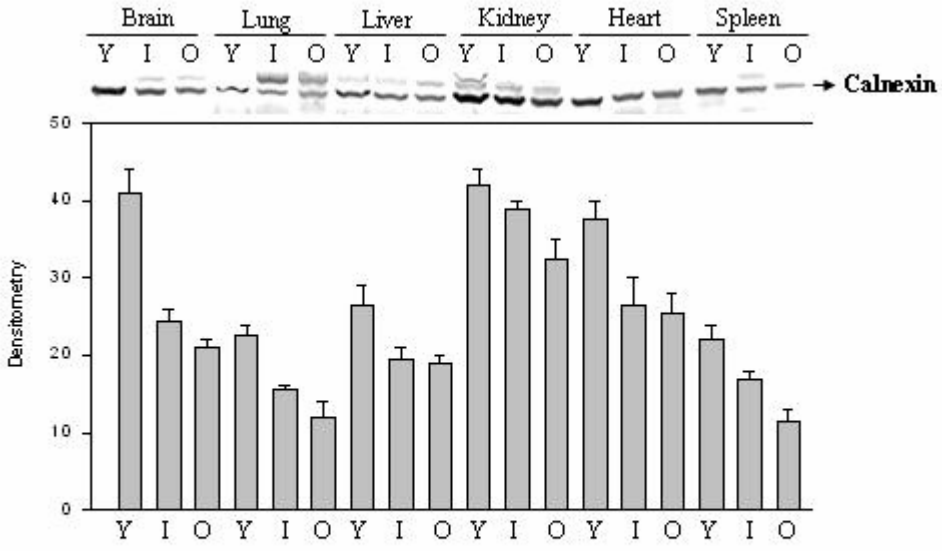
Brain
Lung
Liver
Kidney
Heart
Spleen
Y I O Y I O Y I O Y I O Y I O Y I O
Calnexin
Densitometry
50
40
30
20
10
0
Y I O Y I O Y I O Y I O Y I O Y I O

Fig 5.2

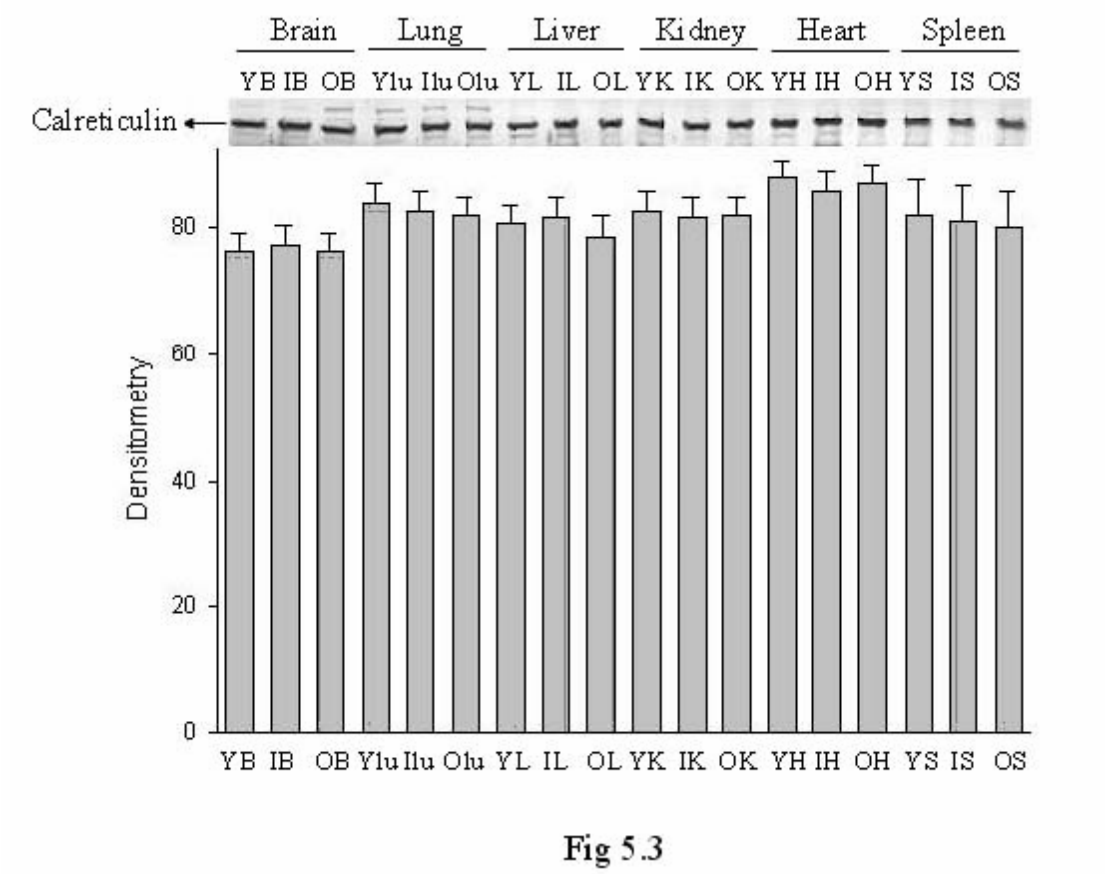

Fig 5.3

Fig 5.3: Immunoblot analysis of age-related changes in the levels of calreticulin in various rat tissues.

Immunoblot analysis for calreticlulin was done as described in methodology using 50 μg of ER enriched fractions of brain (B), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from suckling (Y), 1 month old (I), and old (O) wistar rats. The densitometric analysis for each individual lane is depicted as a vertical bar diagram below the blot.

5.2.2: Age-related changes in the activation status of ER stress transducers IRE-1 and PERK: We have observed a decline in the level of spliced XBP1 mRNA in the brain, lung, and liver tissues of aged rats (**Fig 5.4,** lanes YB vs OB Ylu vs Olu and YL vs OL)**.** During ER stress, the release of BiP from the regulatory domains of IRE-1 leads to oligomerization, autophosphorylation and the subsequent activation of IRE-1 endoribonuclease and kinase activity in its cytoplasmic domain (Tirasophon et al. 1998)**.** Active endoribonuclease splices XBP-1 mRNA in mammals. The unspliced human XBP-1 mRNA contains an ORF1 coding for 261 amino acids and ORF2 encoding 222 amino acids that partially overlaps with ORF1. Splicing of the intron results in a translational frame shift at amino acid 165 in ORF1 and also removes the C-terminal 97 amino acids from ORF1 and adds on 212 amino acids of ORF2 to the N-terminal of ORF1. The splicing events result in the formation of a 376 amino acid XBP-1 protein, with a novel C terminus that acts as a potent bZIP transcription factor, in contrast to a 261-aminoacid XBP-1 protein encoded by unspliced XBP-1 mRNA (Yoshida et al. 2001). The decline in the levels of spliced XBP-1 mRNA in aged tissues suggests that with age, i) the endoribonuclease activity of IRE-1 declines, and/or ii) there is decline in the efficiency of activation of IRE-1.

ATF4 is a transcript that is upregulated at translational level during physiological conditions of eIF2α phosphorylation and is downstream to eIF2α phosphorylation. Although the levels of ATF4 mRNA do not significantly alter with age (**Fig 4.10**), we have observed high levels of ATF4 protein (**Fig 4.9**) in young tissues correlating with the levels of eIF2α phosphorylation (**Fig 3.7**) inspite of low levels of eIF2α kinases PKR and PERK (**Fig 4.1 and 4.2**), suggesting that in the aged rat tissues, the eIF2α kinases PERK and PKR are negatively regulated. PERK is an ER resident eIF2α kinase and an ER stress transducer hence our observations suggest that even though the levels of PERK increase with age (**Fig 4.2**), its activity is negatively regulated in aged tissues and it may compromise the ER-stress response in aged tissues. Hence these observations suggest that young rat tissue experience mild physiological ER stress due to their growth requirements and possess efficient ER stress response mediated adaptive signalling pathways initiated by IRE-1 and PERK, as adjudged by the XBP1 mRNA splicing (**Fig 5.4**) and eIF2α phosphorylation (**Fig 3.7)**. In old tissues such adaptive ER stress signalling pathways mediated by IRE-1 and PERK may be compromised.

Fig 5.4: Analysis of the levels of XBP-1 mRNA splicing in young (Y) and aged (O) rat brain (B), lung (lu), and liver (L) tissues.
The XBP-1 splicing assay was performed as described in methodology. The cDNA prepared from the total RNA extracted from various rat tissues was amplified using XBP-1 specific primers that span regions that undergo splicing. The resultant 600 bp PCR product from unspliced XBP-1 mRNA can be cleaved by Pst-1, whereas, the splicing of the 26 nt intron results in the loss of Pst-1 cleavage site in spliced XBP-1 mRNA, and hence the resultant 574 bp PCR product cannot be cleaved by Pst-1. The apparent levels of Pst-1 cleaved and uncleaved fragment would thus give an idea about the relative levels of IRE-1 mediated XBP-1 mRNA splicing. The levels of amplified PCR fragment generated using tubulin primers is shown below, indicating equal amounts of cDNA was used for the above analysis in all tissues. Lanes are as follows: YB, Ylun, and YL stand for young brain, lung, and liver where as OB, Olun, and OL stand for old brain, lung, and liver. Lane M, represents 1 kb DNA ladder. The Pst-1 cleaved fragments, unspliced XBP1 (uXBP1 ~ 600 bp) and spliced XBP1 (sXBP1 ~ 574 bp) are depicted by arrows in the figure.

Fig 5.5: Analysis of NFκB activation by EMSA in young and aged rat tissues.
NFκB activation was assessed by incubating radiolabelled NFkB binding sequence oligonucleotide with 10 μg of nuclear extract obtained from young suckling (Y) and old (O) rat brain (B), lung (lu), liver (L) and kidney (K) tissues as described in methodology. Then the reaction mix was separated on 5% native PAGE, the gel dried and exposed to phosphorimager. The lanes in the figure as follows: YB, Ylu, YK and YL stand for brain, lung, liver and kidney nuclear extracts obtained from young suckling rats. OB, Olu, OL and OK stand for brain, lung, liver and kidney nuclear extracts from aged rats (> 12 months). A control reaction with excess unlablelled probe and young liver nuclear extract, done to ascertain the specificity of the reaction was run in lane C.

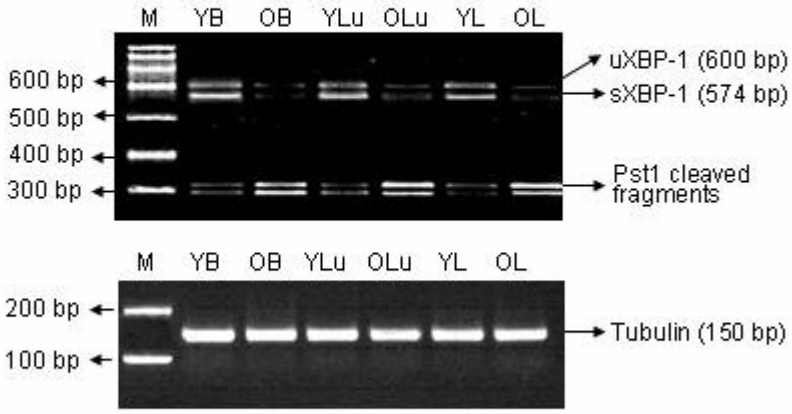
M
YB
OB
YLu
OLu
YL
OL
600 bp
500 bp
400 bp
300 bp
uXBP-1 (600 bp)
sXBP-1 (574 bp)
Pst1 cleaved fragments
M
YB
OB
YLu
OLu
YL
OL
200 bp
100 bp
Tubulin (150 bp)

Fig 5.4

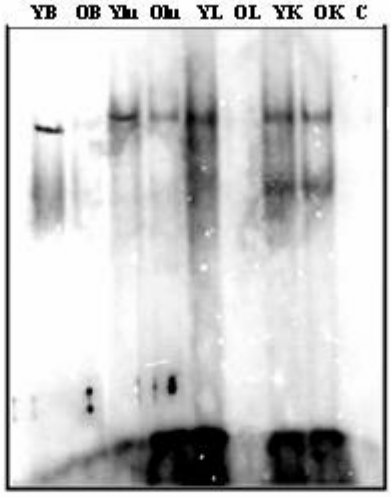
YB
OB
Yku
Oku
YL
OL
YK
OK
C

Fig 5.5

In higher eukaryotes, active IRE-1 is implicated to recruit scaffolding protein TRAF2 that results in the interaction between IRE-1 and IKK leading to the activation of NFκB, a transcription factor involved in the immune and inflammatory stress responsive pathway (Hu et al 2006). The phosphorylation of eIF2α is also implicated in NFκB activation (Jiang et al. 2003; Deng et al 2004). Hence, we also analysed the activation of NFκB in some tissues of young and old rats. We observed an enhanced activation of NFκB in young brain, lung and liver tissues with respect to their older counterparts (**Fig 5.5,** lanes YB vs OB, Ylu vs Olu, YL vs OL and YK vs OK). Our observations correlate positively to the levels of eIF2α phosphorylation (**Fig 3.7**) and IRE1 activation (as accessed by XBP1 splicing, **Fig 5.4**) in young rat tissues and justifies the above notion that active IRE-1 and eIF2α phosphorylation may independently trigger NFκB activation.

5.2.3: Enhanced activation and expression of proapoptotic proteins JNK and CHOP in aged rats: We have observed an age-dependent increase in the levels and phosphorylation status of JNK2 in all the rat tissues analyzed (**Fig 5.6 and 5.7**)**.** Our results correlate positively with earlier reports that linked enhanced expression of proapoptotic proteins CHOP and JNK in aged hepatocytes sensitizing them to oxidants and ER stressors (Ikeyama et al. 2003; Li and Holbrook, 2004). Active IRE-1 is implicated in the activation of the proapoptotic JNK kinase through the recruitment of scaffolding protein TRAF2 and ASK1. IRE-1 activation and TRAF2 recruitment facilitate the formation of the heterotrimeric complex of IRE-1-TRAF2-ASK1, followed by the subsequent activation of ASK1 (Urano et al. 2000; Nishitoh et al. 1998). ASK1 activates MKK4 and MKK7 that target JNK (Roux and Blenis, 2004). The IRE-1-XBP-1 is the last pro-survival branch of UPR however persistent ER stress may trigger the pro-apoptotic potential of IRE-1 (Szegezdi et al. 2006). It is possible that the IRE-1 interacting proteins, JIK and JAB1, play a role in stimulating the pro- and anti-apoptotic affects of IRE-1. JIK facilitates IRE-1-TRAF2 interaction, whereas JAB1 impedes the interaction. However, during severe ER stress IRE-1-JAB1 interaction is diminished facilitating IRE-1 interaction with TRAF2-ASK1 (Szegezdi et al. 2006). Therefore the high levels of phosphorylated JNK (**Fig 5.7**) observed in aged rat tissues suggests that age-associated alterations may induce a lasting ER dysfunction in aged cells leading to a switch of pro-survival signalling of IRE-1 to that of proapoptotic signalling. In addition to activation of JNK, we also

Fig 5.6 and 5.7: Immunoblot analysis of age-related changes in the levels of JNK 2 (Fig 5.6) and phosphorylated JNK2 (p-JNK2) (Fig 5.7) in various rat tissues.

Immunoblot analysis of JNK2 and phosphorylated JNK2 (p-JNK2) was performed as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant of brain cortex, brain cerebellum, lung, liver, kidney, heart and spleen tissues obtained from young suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The densitometric analysis with respect to the levels of β-actin for each individual lane is depicted as a vertical bar diagram below the blot.

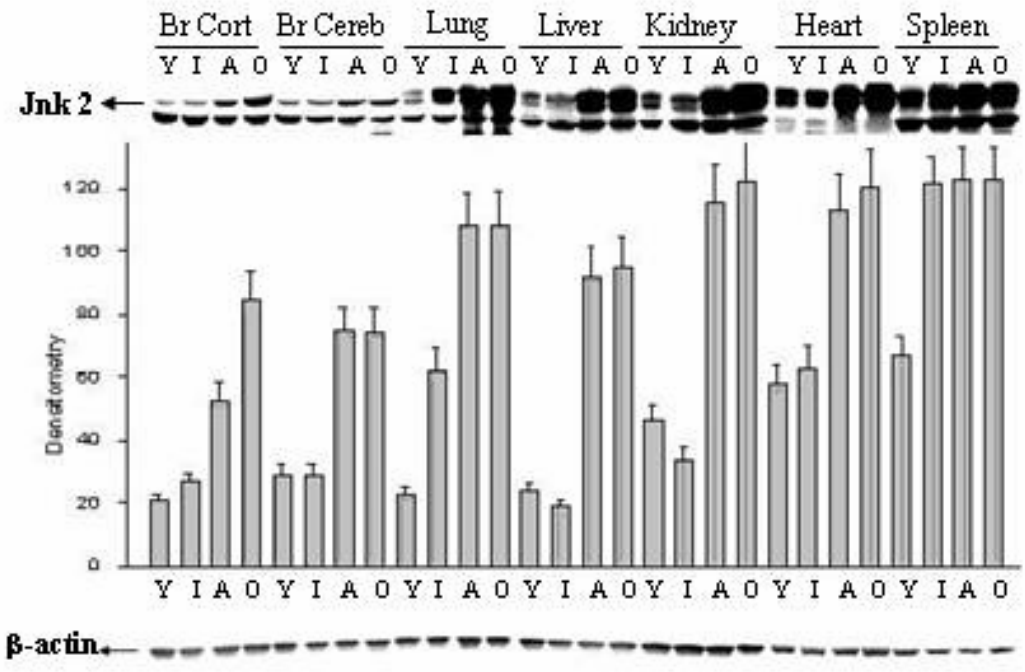
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Y I A 0
Jnk 2
Densitometry
0
20
40
60
80
100
120
β-actin

Fig 5.6

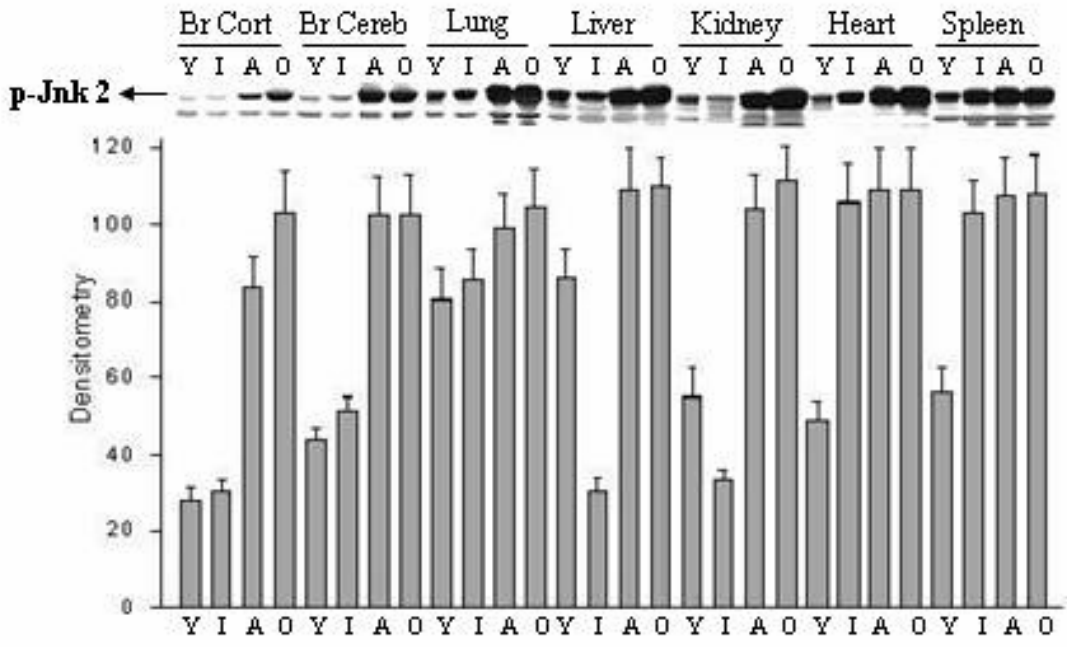
Br Cort
Br Cereb
Lung
Liver
Kidney
Heart
Spleen
Y I A 0
p-Jnk 2
Densitometry
0
20
40
60
80
100
120

Fig 5.7

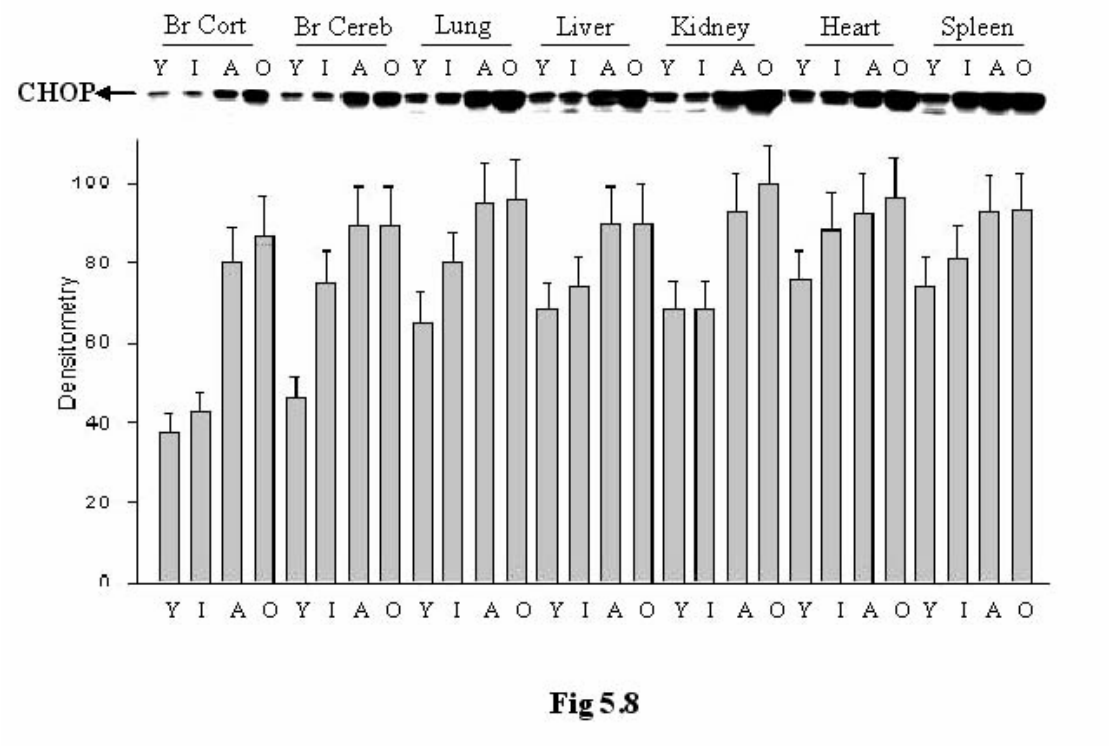

Fig 5.8: Immunoblot analysis of age-related changes in the levels of CHOP in various rat tissues.

Immunoblot analysis for CHOP was done as described in methodology by using 50 μg equivalent protein of post mitochondrial supernatant brain cortex (B Cort), brain cerebellum (Br Cereb), lung (lu), liver (L), kidney (K), heart (H) and spleen (S) tissues obtained from suckling (Y), 1 month old (I), adult (A) and old (O) wistar rats. The densitometric analysis for each individual lane is depicted as a vertical bar diagram below the blot.

observed an age-dependent induction in the levels of another ER-stress associated proapoptotic protein CHOP, in all aged rat tissues analyzed (**Fig 5.8**). CHOP is one the highly induced genes during ER stress and is known to be upregulated by all three ER-stress induced bZIP transcription factors, ATF4, XBP1 and p50ATF6 (Oyadomari and Mori, 2004). Although the eIF2α phosphorylation and ATF4 has been shown to play the most dominant role in CHOP induction, paradoxically, we observed an inverse correlation between ATF4 and CHOP levels (**Fig 5.8 and 4.9**) suggesting that other transcriptional inducers of CHOP, like ATF3, XBP1 and p50ATF6 may play a more dominant role in age-dependent induction of CHOP gene. CHOP is also shown to be induced during oxidative stress and aging is associated with elevated levels of oxidative stress and decline in the efficiency of anti-oxidant response (Holbrook and Ikeyama, 2002). Hence it is also likely that high levels of CHOP in aged tissues may have been induced due to high oxidative stress experienced by them.

Another possibility is that the during persistent ER-stress the GADD34 mediated increment in eIF2α specific phosphatase activity and restoration of translation, may possibly result in enhanced translation of proapoptotic mRNAs, whose products accumulate during prolonged and persistent ER stress (Szegezdi et al. 2006). Since aging is probably associated with progressive ER dysfunction it is likely that high levels of CHOP protein in aged tissues (**Fig 5.8**) is due to an age-dependent increment in eIF2α specific phosphatase activity (**Fig 4.5)** via the induction of GADD34, a cofactor of eIF2α phosphatase (**Fig 4.7)** that facilitates the synthesis of proapoptotic proteins during prolonged stress conditions by restoring translation.

5.3: Conclusions: We observed an age-dependent decline in the levels of ER chaperones BiP and calnexin, in all the rat tissues analysed, suggesting that young tissue experience mild growth related physiological ER stress and the ER-stress mediated adaptive signalling pathways operate efficiently in them. The elevated amounts of spliced XBP1 mRNA, eIF2α phosphorylation and ATF4 levels in young tissues suggest that the adaptive signalling pathways mediated by ER stress transducer IRE1 and PERK operate efficiently in young rats. In contrast, the adaptive signalling activity of these ER stress transducers deteriorates with age. The decline in the levels of eIF2α phosphorylation and ATF4, with a concomitant increase in the eIF2α specific phosphatase activity in aged rat tissues also suggests that the eIF2α phosphorylation mediated adaptive signalling weakens with age. The induction of

proapoptotic CHOP protein and activation of JNK in aged tissues suggests that during aging, the activity of the ER stress induced adaptive signalling pathways diminish and the potential of proapoptotic pathways is bolstered. The increased expression of CHOP may also be due to restoration of translation by GADD34 facilitating the synthesis of proapoptotic proteins like CHOP, whose mRNAs probably accumulate during persistent or prolonged ER stress, like in aging.

Summary:

The major observations of the present thesis are summarised below,

Chronological aging in wistar rats is associated with

- a decline in the levels of translational intiation factors such as eIF4E, eIF2, eIF5, eIF2B, and small ribosomal protein S6.
- a decline in the phosphorylation status of eIF4E, 4EBP1, eIF2α and small ribosomal protein S6.
- a decline in the total polyribosome content and increase in the peaks corresponding to low molecular weight polysomes with a corresponding decline in the high molecular weight polysome peaks.
    - ❖ Overall these observations suggest that, with aging the efficiency of translational intiation, specifically the recruitment of eIF2.GTP.Met-tRNAi^{Met} and mRNA to the 40S ribosomal subunit declines.
    - ❖ Our observations indicate very high levels of eIF4E, S6 and the phosphorylated forms of eIF4E, 4E-BP1 and S6 in young rat tissues which justifies the notion that eIF4E/4E-BPs and S6 kinases function cooperatively in translational control to regulate mammalian cell growth, size and proliferation in response to growth and nutrient stimuli.
- Suprisingly, we observed a decline in the phosphorylation status of eIF2α, a negative regulator of translation during aging and our observation does not correlate with the high rates of translation occurring in young rats
    - ❖ however recent studies have implicated eIF2α phosphrylation in the translational upregulation of growth and stress response associated mRNAs which possess specific *cis*-acting elements in their 5' UTR. Hence we assume thay the high levels of eIF2α phosphorylation

observed in young tissues may probably play a role in stimulating the translation of specifc mRNAs that are pivotal for growth, differentiation and physiological-stress adaptation.

- ❖ in addition the high levels of eIF2B observed in young tissues, suggest that young tissues effectively tolerate toxic levels of eIF2α phosphorylation compared to the aged tissues.

- the levels of eIF2α kinases PERK and PKR increased during aging.
- the eIF2α kinase activity activity declined during aging.
- the eIF2α–specific phosphatase activity increased in aged rat tissues.
- the expression of GADD34 a cofactor of eIF2α specific phosphatase, PP1 incresed with aging
- the expression of ATF4, a down stream target of eIF2α phosphorylation decline with aging
    - ❖ these observations imply that the eIF2α kinase activity is negatively regulated paralled by concomitant increase in eIF2α specifc phosphatase activity during aging, suggesting that aging is associated with a decline in the eIF2α phophorylation mediated modulation of gene expression during stress response.
- the expressioin of ER chaperones BiP and calnexin declines with aging
- the IRE-1 mediated splicing of XBP1 mRNA declines with aging
- the expression of proapoptotic transcription factor CHOP increases during aging
- the activation of proapoptotic kinase JNK inceases during aging
    - ❖ overall these observation indicate that the young tissues are experiencing mild physiological ER stress and the adaptive signaling pathways are operating efficiently in them. Whereas the adaptive signalling mediated by ER stress transducers PERK and IRE-1 gets compromised and the proapoptotic potential of the ER stress signalling pathways bolstered with aging.

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Reduced eIF2α phosphorylation and increased proapoptotic proteins in aging

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Abstract

A decline in relative levels and phosphorylation of many of the eukaryotic initiation factors (eIFs) including S6, the 40S ribosomal subunit protein in many of the rat tissues during chronological aging is accompanied by elevated levels of eIF2α kinases, such as PKR and PERK, but not their activity. Concomitant with increased eIF2α phosphorylation, young tissues displayed a higher level of eIF2B to tolerate the toxic effect of eIF2α phosphorylation on translation, ATF4, a b-zip transcriptional factor that is produced as part of the gene expression programe in response to eIF2α phosphorylation, and BiP, an endoplasmic reticulum (ER) molecular chaperone and regulator of ER stress sensors. Decline in eIF2α phosphorylation in aged tissues is associated with a higher level of GADD34, a subunit of eIF2α phosphatase, and proapoptotic proteins like CHOP/GADD153 and phospho JNK, suggesting that young tissues possess an efficient ER stress adaptive mechanism that declines with aging.

Keywords: Aging; eIF2α phosphorylation; ER stress; Translation; ATF4; BiP; GADD-34; CHOP

Aging is found associated with a change in gene expression. Microarray analysis of aged heart tissues reveals a transcriptional shift toward carbohydrate metabolism, down-regulation of genes involved in fatty acid metabolism, and the induction in the expression of structural proteins [1]. Translational regulation of eukaryotic mRNAs play a role in development, differentiation, and death. The translation of eukaryotic mRNAs is regulated by several polypeptides and RNAs. However to date, eIF2α, eIF2Bε, eIF4E, eIF4G, S6, eEF1, and eEF2 have been identified as targets for regulatory pathways [2]. In the initiation step of protein synthesis, eIF2, a heterotrimer bound by GTP joins the initiator tRNA and delivers it to 40S ribosomal subunit to form 43S multifactor complex. Messenger RNA that is bound on its 5′ end by eIF4F complex consisting of eIF4A, an RNA helicase and ATPase, 4E, the 5′ cap recognition factor, and, 4G, a scaffolding protein then interacts with eIF3 of the 43S complex. Soon after the recognition of the start codon, eIF5, a GTPase activating protein stimulates the GTPase activity of eIF2 and eIF2.GDP is released [3]. Global translation rates are affected chiefly through changes in phosphorylation–dephosphorylation of eIF2 and eIF4E that affect the binding of initiator tRNA (Met-tRNAi) and cap-dependent mRNA. Translation of specific RNAs is also modulated by RNA-binding proteins that bind the cis-acting elements in the 5′ UTR of mRNAs [4,5].

Phosphorylation of eIF4E at ser^{209} by Mnk1 and its interaction with the repressor 4E-binding proteins (eIF4E BPs) determine its activity to bind the 5′ cap structure and to maintain eIF4F integrity. Unphosphorylated 4E-BPs' are competitive inhibitors of eIF4G binding to eIF4E and inhibit cap-dependent translation [6]. Mammalian target of rapamycin (mTOR) kinase that is activated in response to nutrients and growth stimuli phosphorylates 4E-BPs, and is also implicated in S6 kinase activation and phosphorylation of S6. The latter modulates the translation of mRNAs possessing 5′ terminal oligopyrimidine tracts (5′ TOP mRNAs) that code for ribosomal proteins

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and other components of translational machinery [6]. In contrast, phosphorylation of ser^{51} residue in eIF2α inhibits the activity of eIF2B, the factor that recycles eIF2.GDP to eIF2.GTP, reduces the ability to join Met-tRNAi and attenuates translation in general [2,7]. Phosphorylation of eIF2α plays a role after birth as demonstrated by knock-in experiments [8] and is a point of convergence to diverse stress-induced signaling pathways. Its ability to promote adaptive or apoptotic response is dependent on the coincident signaling activities [9].

As of date four eIF2α kinases are well characterized. These are: heme-regulated inhibitor (HRI), double-stranded RNA-dependent kinase (PKR), GCN2, and an endoplasmic reticulum (ER) transmembrane-resident kinase (PERK) that recognize the various stressful conditions such as iron or heme-deficiency, amino acid starvation, viral infection, and accumulation of unfolded proteins, respectively [4]. The decline in general translation through eIF2α phosphorylation protects the cells from unfolded protein accumulation in cytosol or in ER by relieving the burden of protein folding chaperones [10,11], saves amino acid pools for important functions [12] and provides resistance to oxidative stress [13,14]. As part of eIF2α phosphorylation induced gene expression programe, transcripts with multiple uORFS like mammalian ATF4 and yeast GCN4 are upregulated which in turn facilitate the synthesis of several genes involved in amino acid synthesis, transport, and redox chemistry [3,4,15]. In addition, translation of certain mRNAs that contain internal ribosome entry sequences like CAT1 and growth factor mRNAs such PDGF-2, VEGF, and c-Myc have been correlated to enhanced eIF2α phosphorylation [16]. Cellular regulators like type1 protein phosphatase, chaperones like p58IPK and expression of GADD34, a stress-induced subunit of the eIF2α holophosphatase restore translational block imposed by eIF2α phosphorylation [17–19]. Inhibition of eIF2α phosphatase specifically by a compound like salubrinal protects cells from ER stress-induced apoptosis and HSV (Herpes simplex virus) infection [20].

Chronological aging in rats is accompanied by increased oxidative stress and reduced stress tolerance to oxidative injury, reflecting a decreased ability to activate certain prosurvival signals in response to the insults [21,22]. Hepatocytes of old rats display greater sensitivity to ER stress than young cells which is linked to elevated expression of proapoptotic GADD 153 and JNK activation [23,24]. Our studies reveal a decline in the levels and phosphorylation status of many of the eIFs is accompanied by a decline in the abundance of ribosomes as estimated from S6 ribosomal protein in aged tissues. We also report here that a decline in eIF2α phosphorylation in aged tissues is accompanied by a decline in the activity of eIF2α kinases, elevated levels of GADD-34, and proapoptotic proteins like CHOP and phospho JNK. The enhanced eIF2α phosphorylation is strongly correlated to enhanced levels of ATF4 and BiP in young tissues, suggesting that the adaptive ER stress response pathways are well maintained. In old rats, a decline in general translation accompanied by a decline in the phosphorylation of eIF2α may facilitate a switch from the synthesis of prosurvival to proapoptotic proteins that sensitizes them to any stress-induced death signaling pathways.

Materials and methods

Materials

Wistar rats were kept under standard conditions of light and temperature and fed *ad libitum* access on commercial rat chow and water. All experiments were carried out according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India, guidelines. The antibodies are obtained from Cell Signaling Technologies (eIF4E, phospho-eIF4E, phospho-4EBP's, S6, and phospho-S6), Research Genetics (phospho-eIF2α), Calbiochem (GRP-78/BiP), Sigma (β-actin), and Santa Cruz (PKR, eIF2Bε, CHOP, and phospho-JNK). Polyclonal ATF4, monoclonal eIF2α, and PERK were kind gifts obtained from Drs Wek, Indianapolis, Chen from MIT and Randal Kaufman from the University of Michigan Medical Centre, respectively. All other materials were obtained from Sigma. Recombinant baculovirus expressed eIF2α was obtained as described previously from this laboratory [7]. PKR-GST clone was a kind gift from Prof. Bryan G Williams, Cleaveland Clinic Foundation, Ohio.

Experimental procedure

Rats were sacrificed by decapitation. Tissues were dissected from a group of 4–5 animals of different age groups: suckling (0–4 day old), young (1 month old), adult (~6 months old), and old (>18 months old), flash freezed in liquid nitrogen and stored at −70 °C. The tissues were homogenized in 1:4 volumes of buffer H consisting 50 mM Hepes/KOH, pH 7.55, 140 mM potassium acetate, 4 mM magnesium acetate, 2 mM DTT, 1 mM EDTA, 0.5 mM EGTA, 100 μM PMSF, 10 μg/ml each of aprotinin, leupeptin, pepstatin, 50 mM sodium fluoride, 20 mM β-glycerophosphate, 20 mM sodium molybdate, 2 mM benzimidine, and 0.32/0.032 M sucrose for brain/other tissue respectively. The homogenate was spun at 11,000*g* for 15 min to obtain the postmitochondrial supernatant (PMS). ER-enriched pellet was obtained by centrifuging the PMS preparation made in buffer H containing 250 mM sucrose at 30,000 rpm for 30 min. The ER-enriched pellets were dissolved in buffer containing 8 M urea, 4% CHAPS, and 100 mM DTT. Bicinchoninic acid reagent from Sigma was used to estimate protein. Proteins of the PMS fraction or ER-enriched fractions were separated by 10% SDS–PAGE and transferred to a nitrocellulose membrane for immunoblot analysis as described previously [7]. All experiments were repeated at least three times with different batches of extracts and the gels were scanned in Biorad Densitometer, GS 800 and analyzed by using Quantity One Software (data not shown).

In vitro phosphorylation of eIF2α. The eIF2α kinase activity of young and old rat brain extracts was performed in the presence of recombinant human eIF2α or mouse PKR. We also analyzed the inhibition in PKR-mediated human eIF2α phosphorylation in vitro by supplementing different concentrations of 0–80% ammonium sulfate cut fractions obtained from young and old rat brain extracts. Typically the phosphorylation of purified recombinant human eIF2α (~50 ng) by mouse PKR (~30 ng) was carried out at 30 °C for 15 min in the presence of 100 μM unlabeled ATP, or 30 μM unlabeled ATP and 20 μCi of γ-[^{32}P]-labeled ATP in a standard reaction mixture of 25 μl containing 80 mM KCl, 2 mM $MgCl_2$, 1 mM DTT, in 20 mM Tris–HCl, pH 7.8. Reactions were supplemented either with young or old tissue extracts or different concentrations of ammonium sulfate cut fractions. The reactions were terminated by the addition of SDS sample buffer and the phosphorylation of eIF2α was analyzed by a phosphospecific anti-eIF2α antibody or by phosphorimager as described previously [7].

Results and discussion

Initiation factors and their phosphorylation are reduced during aging

Relative levels of several eukaryotic initiation factors (eIFs) viz., eIF2α, eIF2Bε, eIF5, eIF4E (Fig. 1), and S6, a 40S ribosomal protein (Fig. 1), and also the phosphorylation of eIF2α, eIF4E, S6, and eIF4E binding proteins (eIF4E BPs) (Fig. 2) reduced during aging in different rat tissues. The reduction in the abundance of ribosomes as measured from S6 levels and in translational factors during aging is consistent with the previous observations that translation decreases with aging [1,25]. The finding also suggests that young tissues are experiencing some kind of a stress. Young rats also have higher levels of eIF2B as judged by the analysis of eIF2Bε in all the tissues (Fig. 1) to withstand the toxic effects on translation imposed by enhanced levels of eIF2α phosphorylation. These findings are also consistent with the idea that eIF2α phosphorylation plays an important role soon after birth than during embryonic development as has been substantiated by knock-in experiments [8]. Enhanced eIF2α phosphorylation may also promote the translation of many genes that have upstream inhibitory small ORFs or IRES involved in the control of cellular growth, differentiation, and death [5,26].

Phosphorylation of eIF2α is inversely correlated to the levels of eIF2α kinases in young rats

Interestingly a correlation is not observed during aging between eIF2α phosphorylation and the levels of two of the eIF2α kinases, PKR and PERK that we have analyzed here (Fig. 1). The eIF2α kinases phosphorylate the same residue in eIF2α however their cellular localization, mechanism of activation and the regulatory mechanisms are different. The levels of eIF2α kinases increase with age (Fig. 1) and are inversely proportional to eIF2α phosphorylation thereby suggesting that their activity is negatively regulated during aging. Hence we have analyzed the activity of recombinant mouse PKR to phosphorylate recombinant human-eIF2α (Fig. 4) in the presence of young and old brain tissue extracts (Fig. 4A) and alternatively in the presence of increasing concentration of 0–80% ammonium sulfate concentrated fractions of young and old brain extracts (Fig. 4B). Consistent with the notion that the eIF2α kinase activity is negatively regulated during aging, we observed here that (a) endogenous and exogenously supplemented recombinant eIF2α is efficiently phosphorylated by exogenously added recombinant PKR in the young extracts than in extracts obtained from old rats (Fig. 4A, lanes 1 and 2 vs 3 and 4), and (b) the phosphorylation of human eIF2α by recombinant PKR declined by addition of increasing concentrations old rat brain extract and was unaffected by the addition of young rat brain extract (Fig. 4B, lanes 1–3 vs 4–6). This finding is consistent with a relatively recent report indicating that PKR and, p58IPK, a molecular chaperone and cellular inhibitor of eIF2α phosphorylation are high in aged tissues [27]. In addition, induction of GADD-34 that dephosphorylates eIF2α in the late stages of ER stress response can relieve the translational block imposed by eIF2α phosphorylation and facilitates the synthesis of proapoptotic proteins like CHOP 153 [18,19]. Consistent with these reports in ER stressed cells, we also

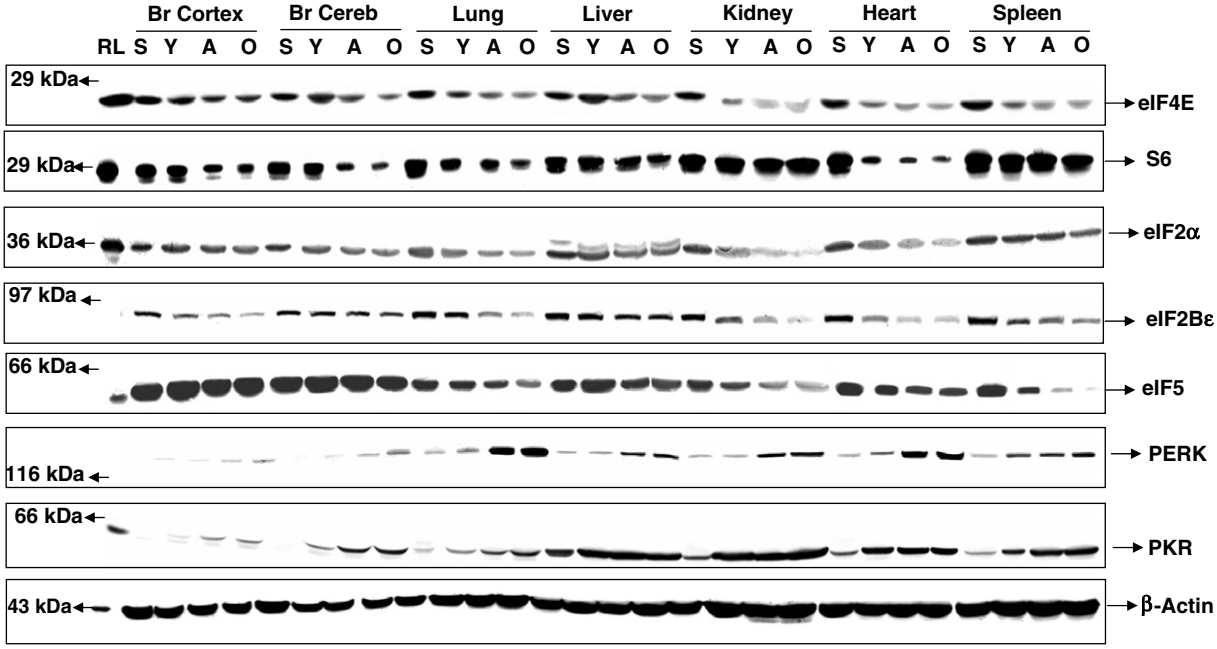

Fig. 1. Levels of eIF4E, S6, eIF2α, eIF2Bε, eIF5, PERK, and PKR in the postmitochondrial supernatant (PMS) fractions of different tissues obtained from suckling (S), young (Y), adult (A), and old (O) wistar rats. Approximately 50 μg of the PMS fractions were separated by 10% SDS–PAGE and the proteins were identified with the respective antibodies as described in Materials and methods. The figure is an immunoblot. RL, reticulocyte lysate was loaded in the first lane as a positive control. β-Actin was used as a loading control in all analyses.

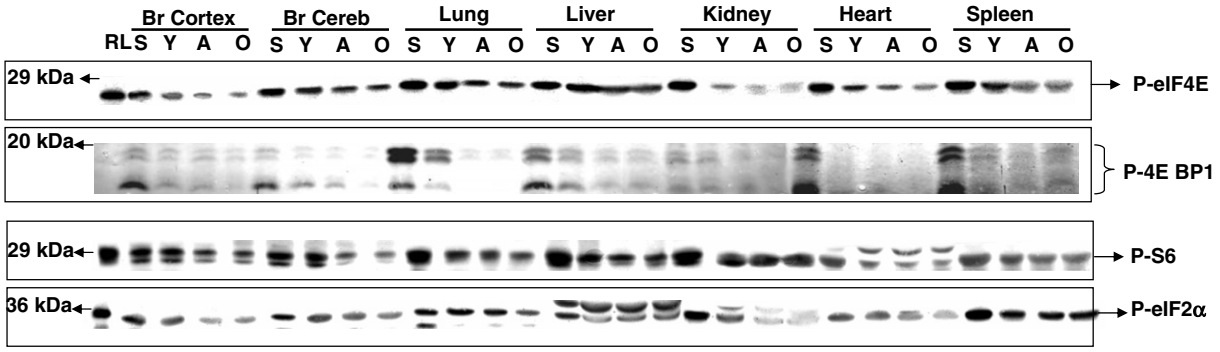

Fig. 2. Levels of phosphorylated eIF4E, eIF4E BPs-1, S6, and eIF2α in the postmitochondrial supernatant (PMS) fractions of different tissues obtained from suckling (S), young (Y), adult (A), and old (O) wistar rats. Approximately 50 μg of the PMS fractions were separated by 10% SDS–PAGE and the proteins were identified with the respective phosphospecific antibodies as described in Materials and methods. The figure is an immunoblot. RL, reticulocyte lysate was loaded in the first lane as a positive control.

observed here that GADD-34 increases during aging (Fig. 3) and is concomitant with the decline in eIF2α phosphorylation.

Prosurvival proteins ATF4 and BiP decrease, and proapoptotic proteins CHOP and phospho-JNK are elevated in aged tissues

Further we observed a strong correlation between increased eIF2α phosphorylation in young tissues to the levels of ATF4 (Fig. 3). Enhanced ATF4 levels insure the supply of amino acids for protein and glutathione biosynthesis, and protects cells against oxidative stress [14]. ATF4 has also been implicated to play a role in memory formation [28]. Further an analysis of BiP, an ER-chaperone and a master regulator of unfolded protein response in the ER [29], indicates that it is relatively high in young tissues than in old tissues (Fig. 3) thereby suggesting that the adaptive ER stress response pathways operate more efficiently in young tissues. In unstressed cells, BiP binds to the ER luminal domains of the three well-known ER stress sensors and transmembrane proteins: PERK (eIF2α kinase), Activated transcription factor-6 (ATF-6), and Inositol-requiring enzyme-1 (IRE-1). In response to the accumulation of unfolded proteins in ER, BiP is released from the stress sensors and binds to unfolded proteins. The release of BiP activates PERK that phosphorylates eIF2α and inhibits general translation and is followed by the activation of ATF6 and IRE-1. While phosphorylation of eIF2α stimulates ATF4 translation, ATF6 moves to golgi and is processed by proteases to produce active ATF6 that moves to the nucleus to induce the expression of XBP-1, CHOP, and chaperones. Active IRE-1, a bifunctional enzyme containing kinase and endoribonuclease activities removes a 26 intron sequence from XBP-1 that triggers the genes for protein degradation, p58IPK, and

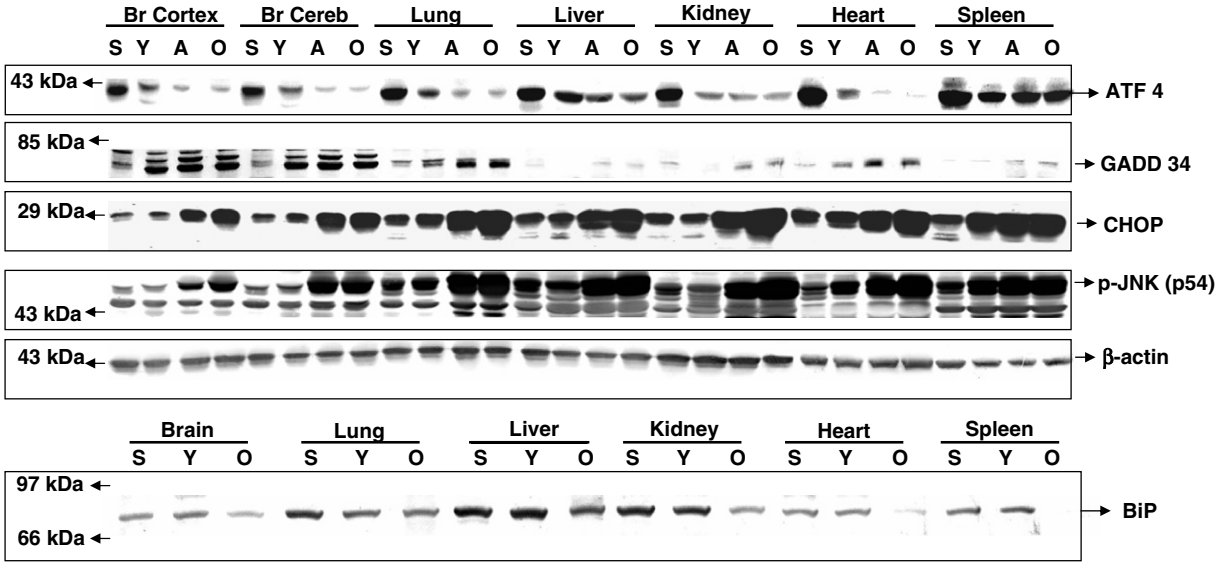

Fig. 3. Levels of ATF4, GADD34, CHOP, and phosphoJNK (p54) in the postmitochondrial supernatant (PMS) fractions and BiP in the ER-enriched fractions of different tissues obtained from suckling (S), young (Y), adult (A), and old (O) rats. Approximately 50 μg of the PMS or ER-rich fractions were separated by 10% SDS–PAGE and the proteins were identified with the respective antibodies as described in Materials and methods. The figure is an immunoblot.

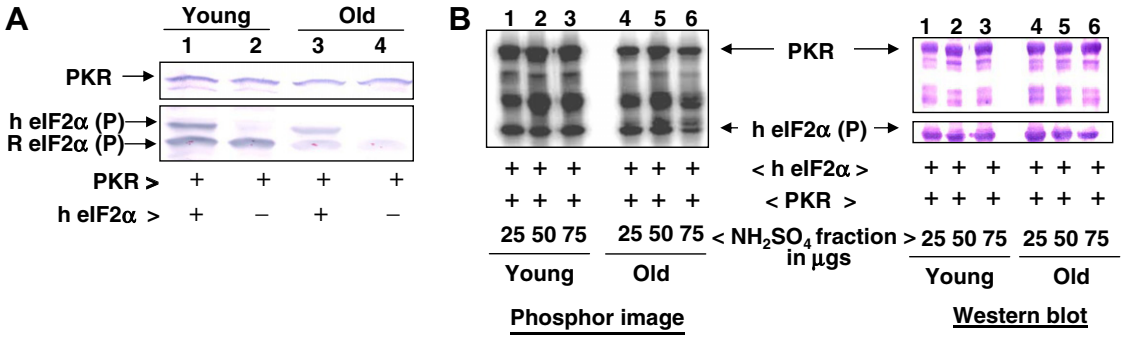

Fig. 4. Phosphorylation of eIF2α in vitro by recombinant PKR in the presence of tissue extracts. (A) Phosphorylation of recombinant human eIF2α (~50 ng) in the presence of young and old brain extracts and also the phosphorylation of the endogenous eIF2α of the brain extracts obtained from young and old rats was carried out by purified recombinant GST-tagged mouse PKR (~30 ng) as described in Materials and methods. The figure is a Western blot indicating the levels of PKR and phosphorylated human and endogenous eIF2α (h eIF2α or R eIF2α). The various lanes are as follows: lane 1, young brain extract + h eIF2α + PKR; lane 2, young brain extract + PKR, lane 3, old brain extract + h eIF2α + PKR, and lane 4, old brain extract + PKR. (B) Phosphorylation of recombinant human eIF2α in vitro by recombinant PKR in the presence of 0–80% ammonium sulfate concentrated tissue extracts. Left side of the panel represents phosphor image indicating the phosphorylation of recombinant human eIF2α (h eIF2α, lower band as shown) and the autophosphorylation of recombinant PKR (upper band as shown) in the presence of different concentrations of ammonium sulfate fractions of the brain extracts obtained from young and old rats. Phosphorylation was carried out in the presence of labeled γ-[^{32}P]ATP as described in Materials and methods. (B) Right side of the panel is a Western blot of the phosphor image probed by PKR and phosphospecific anti-eIF2α antibodies. The various lanes are as follows: lane 1, PKR + h eIF2α + 25 µg young brain extract; lane 2, PKR + h eIF2α + 50 µg young brain extract; lane 3, PKR + h eIF2α + 75 µg young brain extract; lane 4, PKR + h eIF2α + 25 µg old brain extract, lane 5, PKR + h eIF2α + 50 µg old brain extract, and lane 6, PKR + h eIF2α + 75 µg young brain extract.

chaperones [29,30]. Further BiP expression is translationally regulated in the unfolded protein response [31,32].

Phosphorylation of eIF2α and expression of ATF4 are the initial events that occur in response to unfolded protein accumulation in the ER. Consequent activation of other ER stress sensors like ATF6 and IRE-1 results in the synthesis of transcriptional factor like CHOP or GADD153 that in turn promotes the synthesis of GADD34, the eIF2α phosphatase. Analysis of CHOP deleted cells lead to the conclusion that CHOP induces oxidation in the ER and death by promoting protein synthesis through the dephosphorylation of eIF2α mediated by GADD34 [33]. Earlier studies with cultured cells have shown a decline in the tolerance to environmental insults, proliferative capacity, and enhanced sensitivity to ER stress with age [21–24]. Consistent with these earlier findings in stressed cells, BiP and ATF4 levels (Fig. 3) are reduced and, CHOP/GADD153 and phospho JNK (Fig. 3) levels are enhanced during chronological aging. These findings therefore suggest that ER-stress induced eIF2α phosphorylation mechanism is operating very efficiently in young tissues. The idea that reduced eIF2α phosphorylation promoted by proteins like p58IPK or GADD34 can relieve translational block imposed by eIF2alpha phosphorylation and facilitate the synthesis of proapoptotic proteins during persistent stress is consistent with the elevated levels of CHOP and phospho JNK in old tissues. The mild ER stress in young rats may induce hormesis.

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During my PhD tenure, we have also written a review entitled “Endoplasmic Reticulum: Stress, Signaling and Apoptosis”. The review has been accepted for publication in the *Current Science* journal, published by Current Science Association in collaboration with Indian Academy of Sciences. The abstract of the review is shown below.

Endoplasmic Reticulum: Stress, Signaling and Apoptosis.

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The synthesis, folding and processing of the secretory/membrane proteins by the endoplasmic reticulum (ER) requires the functioning of ER chaperones, maintenance of ER calcium pools, and an oxidative environment. Disruption of the ER functioning elicits an adaptive signaling cascade called the unfolded protein response (UPR). UPR is triggered by the activation of ER transmembrane proteins and modulated by ER chaperones. It comprises of, general inhibition in protein synthesis; induction of ER chaperones; and components of ER-associated protein degradation. UPR is implicated in development, disease and in virus infection. Through unknown mechanisms, an irreparable ER stress may bolster the proapoptotic potential of UPR, with a subsequent decline in the adaptation capabilities, initiating cell death. This review highlights the mammalian UPR signaling pathways in general, and the major players implicated in ER stress-induced apoptosis.