Role of αB-crystallin in Stress and Differentiation

THESIS

Submitted for the degree of

DOCTOR OF PHILOSOPHY

To

Jawaharlal Nehru University

New Delhi

Bhairab N. Singh

Centre for Cellular and Molecular Biology

Hyderabad

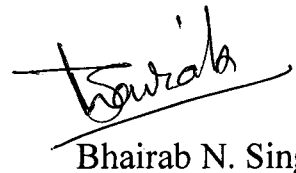
2009

To my father

CERTIFICATE

The research work embodied in this thesis has been carried out at the Centre for Cellular and Molecular Biology, Hyderabad, India. This work is original and has not been submitted in part or full for any other degree or diploma of any other university.

Dr. Ch Mohan Rao
Supervisor



Bhairab N. Singh

Acknowledgements

"The secret to a rich life is to have more beginnings than endings. Just living is not enough. One must have sunshine and freedom."

The above quote explains my entire tenure of five years at Centre for Cellular and Molecular Biology as a PhD student. I take this opportunity to thank my advisor Dr. Ch Mohan Rao for his guidance, support and the freedom. My association with Dr. Mohan Rao has been a great learning experience at both professional level as well as personal level. It's a pleasure to work under the supervision of Dr. Mohan Rao, where I got the freedom of planning and executing the experiments of my own. He has always supported and encouraged me for better learning and understanding the scientific concepts.

I would like to thank Dr. Sridhar Rao, who has been associated with the project. His timely suggestions and critical comments made my job easier. I have been fortunate to have Dr. Ramakrishna Murti, who has been very helpful during the tenure. He taught me the art of scientific writing and given interesting insights whenever I needed. My interactions with Dr. Raman and Dr Srinivas have been very informative and creative. I would like to thank Dr. Madhusudan Rao, Dr. Yogendera Sharma and Dr. Vijaya Gopal for their support and help. I thank Dr. Glembostki for his kind gift of HA-3A-alphaB-crystallin mutant constructs.

I would like to thank Ms Nandini for my experiments with confocal microscopy. She has been very supportive and informative during my interaction with her. I am thankful to Amit Adhikari for teaching me the art of cell biology during my initial days of PhD. My cell biology discussion with Amit, TK Prasad and Aftab has been very helpful and encouraging. I am very grateful to the members of west wing 1st floor; with whom I shared my happiness and sorrow during my stay at CCMB. I thank my lab members namely Abhay, Aftab, Dev, Prabhu, Abhishek, Saad, Abdullah and Rama for creating scientific ambience in the lab and helping me whenever I needed. I would like to thank Sridhar, Ramki, Aftab and Abdullah for taking the pain in critically reading and editing the thesis.

I thank my friends Pavan, Bhuvan, Ranjan, Shalini, Nikita, Rajender, Zahid, Ram Pariskshan, Renu and Sirisha with whom I shared many social occasions apart from scientific discussion. I would like to thank my seniors Saloni, Tirumal, Waseem, Amit and Faiz for their help and encouragement. I also thank Ram Prasad and Sozi for their help during the work. I acknowledge the service of Hanumanthuji and Late Ehasanji, without their help, it wouldnot have been possible to perform the experiments. I also thank Sayedji

and Venuji for the help and support. I would like to acknowledge Anuradha for her help in official work.

I take this occasion to thank the director Dr Lalji Singh for providing such an excellent world-class facility and maintaining a beautiful scientific environment in CCMB. I thank the tissue culture staff, the instrumentation group, fine-biochemical division and the administrative staff for their help. I thank CSIR for the fellowship during my PhD tenure.

It would be injustice if I don't acknowledge my family members especially my parent, who has been a continuous source of inspiration and motivation I required during odd times of my life. I have been very unfortunate not to have my father with me at this stage of my career. It's with highest gratitude I acknowledge my late father, who had taken the entire burden for me to develop as a person. I thank my in laws for their support and belief. Lastly I would like to thank my son Rohan, my wife Rakhi and my brother Rohit for their support and encouragement. Rohan used to ward-off all my tension and headache with his cute smile and naughty behavior. Rakhi has been very patient and supportive during the difficult times and always helped me in odd times. All I can say is I have become richer in terms of knowledge and experience and it has opened more beginnings than endings in life.

-Bhairab

CONTENTS

SYNOPSIS

Cells and tissues are constantly challenged by exposure to extreme conditions that can cause acute and chronic stress. The survival of an organism depends on its ability to cope with these stress conditions. During stress conditions the labile proteins tend to unfold and the nascent polypeptide chains become more susceptible for erroneous folding. These events result in exposure of hydrophobic surfaces leading to the formation of aggregates. Accumulation of excessive protein aggregates eventually leads to disruption of cellular processes, toxicity and cell death. To ensure productive protein folding and its quality control, cells mainly employ "chaperone-mediated pathways". In addition to assist and maintain correct protein folding, molecular chaperones play important role in several cellular processes. This thesis deals with the *in vivo* role of one such molecular chaperone, αB-crystallin, during stress and differentiation.

Chapter 1 provides contextual overview of the protein folding problem in the complex and crowded environment of the cellular milieu. Given the complexity of the folding process, it is likely that several proteins do not achieve the correct folding and assembly, without assistance. This processes gets further worsen under stress conditions such as high temperature and altered pH. Cells responds to these stresses by selectively expressing a set of proteins called as heat shock proteins or stress proteins. Some of these heat shock proteins act as molecular chaperones. Ron Laskey first used the term "molecular chaperone" in 1978 in a publication describing formation of nucleosomes. Molecular chaperones are a group of proteins, which are found ubiquitously and are involved in assisting proper protein folding, assembly and their maintenance. Molecular chaperones bind to hydrophobic patches exposed either during synthesis or due to stress-induced partial unfolding, and prevent non-specific interaction with other proteins. Most chaperones, thus serve to enhance on-pathway folding and prevent the aggregation process in an ATP-dependent manner. Chapter 1 discusses the mechanism of up-regulation as well as the down-regulation of stress response. Under normal conditions, the activities of heat shock factors (HSFs) remain repressed and localized either in the cytoplasm or in the nucleus in an inert monomeric state. These HSFs are activated upon stress or under different physiological conditions such as differentiation. Activation of HSFs is influenced by intramolecular interactions with its different domains as well as by the phosphorylation at critical serine residues. Activated heat shock factors (HSFs) binds to heat shock elements

(HSEs) of the corresponding genes, present in their promoter region. These HSEs are characterized by multiple adjacent and contiguous inverted repeats of the pentanucleotide motif 5'-nGAAn-3' to which different heat shock factors bind to bring about up-regulation. Upon restoring the normal environmental conditions, the activated heat shock response must be repressed to down regulate the response.

The heat shock proteins are classified according to their molecular masses into six major families, HSP100, HSP90, HSP70, HSP60, HSP40 and small heat shock proteins family. Small heat shock proteins constitute a structurally divergent group and are ubiquitously present in virtually all organisms. Small heat shock proteins (sHSPs), a family of heat shock proteins, gets induced under stress conditions and shown to prevent aggregation in an ATP-independent manner. They share characteristics features such as low monomeric molecular mass, formation of large oligomers and chaperone-like activity. It is important to note that all the sHSPs contain about 80-100 residues long conserved region, called the "alpha-crystallin domain" towards the C-terminal region, and is a hallmark feature of this family. They perform a variety of functions under normal as well as during stress conditions. Importantly, many of the neurological disorders, muscle-related diseases have been shown to be associated with mutations in small heat shock proteins. α-Crystallin, a major eye lens protein, belongs to this sHSP family of molecular chaperones. It is composed of 2 closely related subunits, αA- and αB-crystallin, in a 3:1 ratio. In the lens, the extremely high protein concentration (~ 500 mg/ml) and low protein turnover causes accumulation of post-translational modification that destabilizes protein structure leading to aggregation. The presence of relatively high amount of α-crystallin prevents the aggregation and thus helps in maintaining the lens transparency. The presence of α-crystallin in non-lenticular tissues such as muscle, brain and kidney is indicative of its additional functions. αB-Crystallin acts as a molecular chaperone and prevents the stress-induced aggregation of target proteins *in vitro*. Studies from our laboratory have shown that it undergoes structural transitions at higher temperature thereby exposing or reorganizing the hydrophobic patches to which target proteins bind. α-crystallin exhibits very little chaperone like activity at room temperature, which increases significantly at higher temperature. In addition to the chaperone-like activity, αB-crystallin has been well documented in many cellular processes such cell division, differentiation and apoptosis. The function as well as localization of αB-

crystallin is modulated by its phosphorylation at three serine residues- Ser-19, Ser-45 and Ser-59. Phosphorylation seems to play a critical role in modulating its activity as well.

Chapter 2 describes our results on the role of αB-crystallin in modulating actin filament dynamics in cardiac cells. Disruption of the cytoskeleton and disaggregation of actin fibers are among the most immediate effects of stress such as heat shock. Both ischemia and heat-stress cause extensive damage to the cytoskeleton that includes collapse of the intermediate filament network, disruption of the microtubules, rearrangement and/or disorganization of actin microfilaments. While the level of αB-crystallin is enhanced under these stress conditions, reducing αB-crystallin gene expression using anti-sense strategy leads to disruption of the actin microfilament network, suggesting its importance in cytoskeletal maintenance. Using the H9C2 rat cardiomyoblast cell line as a model system, we show that upon heat stress, αB-crystallin preferentially partitions from the soluble cytosolic fraction to the insoluble cytoskeletal protein-rich fraction. Our confocal microscopic images and the immuno-precipitation experiments demonstrate that it interacts directly with the actin filaments. The appearance of fiber-like morphology and its association with actin filaments is similar in both undifferentiated and differentiated cell types. This association seems to be dependent upon on the phosphorylation of αB-crystallin at Ser-45 and Ser-59 residues as inhibiting its phosphorylation abrogates its association. Treatment of heat-stressed H9C2 cells with the actin depolymerzing agent, cytochalasin B, fails to disorganize actin suggesting that αB-crystallin provides stability to the actin filaments. Our results indicate that αB-crystallin regulates actin filament dynamics *in vivo* and protects cells from stress-induced death. Further, our studies suggest that the association of αB-crystallin with actin helps maintenance of pinocytosis, a physiological function essential for survival of cells.

Chapter 3 discusses our findings on the importance of αB-crystallin in the muscle differentiation process. Skeletal myogenesis, a highly organized and complex process, is strictly regulated both temporally and spatially by the myogenic bHLH transcription factors such as MyoD, Myf5, myogenin, MRF4 and MEF2A. MyoD gets activated upon growth factor withdrawal to induce skeletal muscle differentiation that ultimately leads to the fusion of myoblasts into multinucleated myotubes. αB-Crystallin has been shown to get up-regulated by about 10-fold during muscle differentiation. Earlier reports suggest that αB-

crystallin promoter has at least one MyoD/myogenin binding site (MRF). The mutagenesis and knock out studies on αB-crystallin results in severe loss of muscle phenotype, implying its critical function in muscle maintenance. However, the molecular mechanism of its involvement during differentiation is yet to be deciphered. In order to examine the role of αB-crystallin during muscle differentiation process, we generated stable clones of C2C12 cell lines over-expressing αB-crystallin (CRYAB-C2C12). To our surprise, we find that over-expression of αB-crystallin delays the muscle differentiation program to a significant extent. C2C12 myoblasts over-expressing αB-crystallin (CRYAB-C2C12) displayed defects in cell-cycle exit and showed continued proliferation upon induction of differentiation. An analysis of cell cycle markers revealed that CRYAB-C2C12 cells showed higher and sustained cyclin D1 expression and delay in the p21 (a cell-cycle inhibitor) expression during muscle differentiation. The expression of myogenesis marker, myogenin was also delayed significantly. Further, expression profile of MyoD, a master regulator of muscle differentiation is altered; its expression maxim shifted by 12 hrs in CRYAB-C2C12 cells compared to that of C2C12 cells. We find that upon MG132 treatment, the accumulation of MyoD was more rapid in C2C12 cells compared to that of CRYAB-C2C12 cells suggesting that the synthesis was affected in cells over-expressing αB-crystallin. We find that the apparent half-life ($t_{1/2}$) of MyoD gets decreased to 1.42 h in CRYAB-C2C12 cells in comparison to 2.37 h in C2C12 cells, showing increased turn-over in these cells. Further, using immuno-precipitaion experiments, we show that MyoD gets ubiquitinated at earlier time points of differentiation in CRYAB-C2C12 cells compared to C2C12 cells, correlating its faster ubiquitin-proteasome mediated protein degradation. On the other hand αB-crystallin seems to be highly stable during the differentiation process. We show that the level of αB-crystallin increases gradually with increasing time of differentiation. Our western blot analysis for phosphorylation at Ser-59 and Ser-45 shows that αB-crystallin gets phosphorylated at Ser-59 residue and not at Ser-45 position, thereby indicating a specific role of Ser-59 phosphorylated αB-crystallin. The localization of αB-crystallin remains cytoplasmic with no speckles-like staining in the nucleus of the myotubes as seen in case of myoblasts. Interestingly, we find that the fiber-like morphology at the basal optical sections of confocal images suggesting that αB-crystallin might be assisting the extensive rearrangements and reorganization of the cytoskeletal elements during this process. The results described in this chapter provide an interesting insight into the role of αB-crystallin during muscle differentiation.

Mutations in αB-crystallin have been found to be associated with many of the debilitating diseases such as cataract and myopathies. A point mutation, R120G in αB-crystallin has been shown to be involved in desmin related myopathy (DRM). Studies form our laboratory and that of others have shown that R120G-αB-crystallin has altered structure and significantly reduced chaperone-like activity. We have investigated the effect of this mutation in the process of muscle differentiation. Chapter 4 describes our findings on the effect of R120G-αB-crystallin in the differentiation of C2C12 cells. We have generated stable clones of C2C12 cell lines over-expressing R120G-αB-crystallin (R120G-C2C12) and investigated their ability to undergo the differentiation. We find that R120G-C2C12 cells showed complete loss of their ability to differentiate into myotubes. Our results suggest that the expression profile of p21, myogenin and MyoD were similar to C2C12 cells, indicating that R120G-αB-crystallin mutant does not have any effect in the initiation of differentiation program. Interestingly, however, the mutant leads to an extensive cell death during the differentiation process. The increased cell death appears to be due to enhanced activation of caspase-3 leading to increased apoptosis, however, the mechanistic details are yet to be deciphered.

Chapter 5 summarizes our findings on the *in vivo* role of αB-crystallin during heat stress and differentiation process. Our findings have led to a new understanding of the *in vivo* function of αB-crystallin and provide interesting insights for its diverse functions. This chapter also provides a brief description on the possible future lines of investigation based on the findings described in this thesis.

Publications

1. **Singh BN**, Rao KS, Ramakrishnamurti T, Rangaraj N and Rao CM **(2007).** Association of αB-crystallin, a small heat shock protein, with actin: Role in modulating actin filament dynamics *in vivo*. **J. Mol. Bio. (2007),** 366 (3):756-67.

2. **Singh BN**, Rao KS and Rao CM. αB-crystallin, a small heat shock proteins regulates muscle differentiation through regulation of cyclin D1 and alterations of MyoD stability. **(manuscript communicated)**

3. Adhikari AS, **Singh BN,** Rao KS and Rao CM. αB-crystallin modulate NF-κB activity in a phosphorylation dependent manner and prevent C2C12 from TNF-α induced cytotoxicity. **(manuscript communicated)**

CHAPTER 1
INTRODUCTION

Cell is the basic unit of life. The cellular milieu is composed of many organic as well as inorganic molecules. These various components carry out diverse functions for the cell survival and determine their fate. Of all the components, DNA and proteins play a major role in the cell. Although DNA is the genetic material and contains the code for different proteins, it is the proteins that carry out most of the functions. Linear polypeptide chains, of amino acids, need to fold into unique 3 dimensional-native structures to become biologically active molecules. Hence, attaining the native structure is very critical for its activity and normal functioning.

1.1 Protein folding

Protein folding process is guided by several inter-molecular forces including hydrophobic, electrostatic and hydrogen bonding (Dill, 1990; Rose and Wolfenden, 1993; Pace, 1995; Pace *et al.*, 1996). Anfinsen (1973) had shown that the amino acid sequence of the polypeptide chain contains the information for protein folding (Anfinsen and Harber, 1961; Anfinsen, 1973). Levinthal paradox highlighted the futility of random search by a polypeptide chain to attain native structure as the amount of time required for such search would be astronomically large (Levinthal, 1968). A directed folding, on the other hand, would require much less time; this led to proposals of specific folding pathways (Bryngelson *et al.*, 1995; Dill and Chan, 1997). The folding funnel and energy landscape models that take into account of the thermodynamic as well as kinetic parameters are now being invoked to understand protein folding (Leopold *et al.*, 1992; Wolynes *et al.*, 1995; Dinner *et al.*, 2000). It is to be noted, however, that most of the experimental results used for modeling the folding are obtained from studies carried out on small single domain proteins in dilute solutions.

Unlike *in vitro* conditions, the cellular milieu is very crowded and highly complex. The process of protein folding in the cellular environment is very different from the *in vitro* conditions. In addition, under *in vitro* conditions, the entire length of protein is available for refolding at a time, allowing long range interactions and hydrophobic collapse to occur (Saito, 1989). Inside the cell, however, due to sequential translation process, N-terminal residues enter the cytosol first, followed by other residues and eventually the C-terminus (Jaenicke, 1991). Thus the distant parts of the molecule do not have an opportunity to interact and collapse as can happen *in vitro* conditions. During the translation process, a polypeptide chain exposes the hydrophobic residues, which could

lead to non-specific interactions with other proteins resulting in misfolding and non-functional proteins.

1.1.2 Protein misfolding, aggregation and disease

Given the complexity of the folding process, it is likely that several proteins do not achieve the correct folding and assembly, without assistance. Misfolding and aggregation has been a major concern as it has been shown to be associated with many deleterious diseases. It has been shown that stress conditions such as altered temperature, pH and osmotic pressure lead to partial unfolding and aggregation of proteins (Jaenicke, 1995; Kusumoto *et al.*, 1998).

Protein aggregation has been associated with many disease conditions such as Alzheimer's, cystic fibrosis and prion disease (see review-Muchowski, 2002; Dobson, 2004; Chiti and Dobson, 2006). Thus the correct protein folding and assembly of proteins without the formation of aggregates is critical for cell survival. To ensure productive protein folding and its quality control, cells mainly employ "chaperone-mediated pathways" (Kelley and Georgopoulos, 1992; Hartl and Hayer-Hartl, 2002; Dougan *et al.*, 2002; Deuerling and Bukau, 2004).

1.2 Molecular Chaperones

Cells and tissues are constantly challenged by exposure to extreme conditions that cause acute and chronic stress. As a consequence, survival has necessitated the evolution of stress response networks to detect, monitor and respond to the environmental changes (Morimoto *et al.*, 1990; Baeuerle *et al.*, 1995). Adaptation to these stress conditions, in turn, leads to the activation of heat shock response, leading to the expression of heat shock proteins (Lindquist, 1986). Heat shock response was first discovered in *D. melanogaster*, when the larvae exposed to temperature above optimal growth temperature exhibited selective over-expression of a set of gene (Ritossa, 1962). Some of these heat shock proteins act as molecular chaperones (Ellis, 1987; Ellis and van der Vies, 1991). Molecular chaperones are a group of proteins, which are found ubiquitously and are involved in assisting proper protein folding, assembly and their maintenance (Hartl and Martin, 1995; Boston *et al.*, 1996; Fink, 1999; Nishikawa *et al.*,

2005; Liberek *et al.*, 2008). The term molecular chaperones was first proposed by John Ellis, however, Ron Laskey first used the term in 1978 in a publication describing formation of nucleosomes. Laskey *et al.*, (1978) showed that nucleoplasmin, an acidic nuclear protein, is required for the assembly of nucleosome from DNA and histones. Molecular chaperones can now be defined as a class of proteins that assist in protein folding and assembly and do not form the part of the final structure.

Under stress conditions, the level of heat shock proteins gets enhanced or activated to counter or prevent the accumulation of misfolded proteins (Parsell and Sauer, 1989; Parsell and Lindquist, 1993). Up regulation of heat shock proteins was first observed in *Drosophila melanogaster*, where the puffing patterns were observed in the polytene chromosome upon increasing the temperature (Tissieres *et al.*, 1974). Chromosomal puffs were later demonstrated to be active sites of increased transcription that subsequently led to increased protein synthesis. These proteins expressed upon heat shock are called heat shock proteins or stress proteins (De Maio, 1999). The enhanced expressions of heat shock proteins are brought about by binding of "heat shock factors" (HSFs) to the heat shock elements (HSEs) of the target genes, present in their promoter region (Lis and Wu, 1993; Voellmy, 1994). These HSEs are characterized by multiple adjacent and contiguous inverted repeats of the pentanucleotide motif 5'-nGAAn-3' to which different heat shock factors bind and bring about enhanced expression of heat shock proteins (Pelham, 1982; Fernandes *et al.*, 1994). The binding of HSF leads to changes in the organization of chromatin structure localized to the 5'-flanking regions of the heat shock genes (Wu, 1980; Wu, 1984; Giardiana *et al.*, 1992). HSFs have also been shown to associate with components of the chromatin remodellers and basal transcriptional machinery, to yield inducible heat shock mRNA (Rougvie and Lis, 1988; Brown and Kingston, 1997; Mason and Lis, 1997). Among vertebrates, HSFs 1, 2 and 4 are ubiquitous; whereas HSF3 has been characterized only in avian species (Nakai and Morimoto, 1993; Morimoto, 1998). Among the known HSFs, HSF1 is well characterized compared to other HSFs. Mice lacking HSF1 develop normally, but the fibroblasts derived from these mice show lack of stress-induced transcription of heat-shock genes (McMillan, 1998). In avian cells, lack of HSF3 shows severely compromised induction of the heat shock response even in the presence of HSF1 (Tanabe *et al.*, 1998). It appears that the diversity of HSFs provides redundancy and specialization of stress signals, a means to differentially control the rate of transcription of heat shock genes.

1.2.1 Stress and molecular chaperones

The fate of an organism is decided by its ability to cope with the stress conditions. During stress conditions a set of proteins gets up regulated, and are deployed to prevent the aggregation of misfolded proteins (Landry *et al.*, 1982; Pinto *et al.*, 1991; Martin *et al.*, 1992; Parsell *et al.*, 1994). Under conditions of stress, proteins tend to unfold and thereby expose the hydrophobic residues. Molecular chaperones bind to these exposed hydrophobic patches and prevent non-specific interaction with other proteins. The inducible transcription of heat shock genes is the response to environmental stress, certain physiological and pathophyisiological conditions (Figure 1.1) (Morimoto, 1993; Wu, 1995). Environmental stress includes heat stress, oxidative stress, alteration in pH, exposure to heavy metals, inhibitors of metabolic processes etc. Normal physiological processes such as cell cycle, development and differentiation also lead to enhanced expression of stress proteins (Figure 1.1).

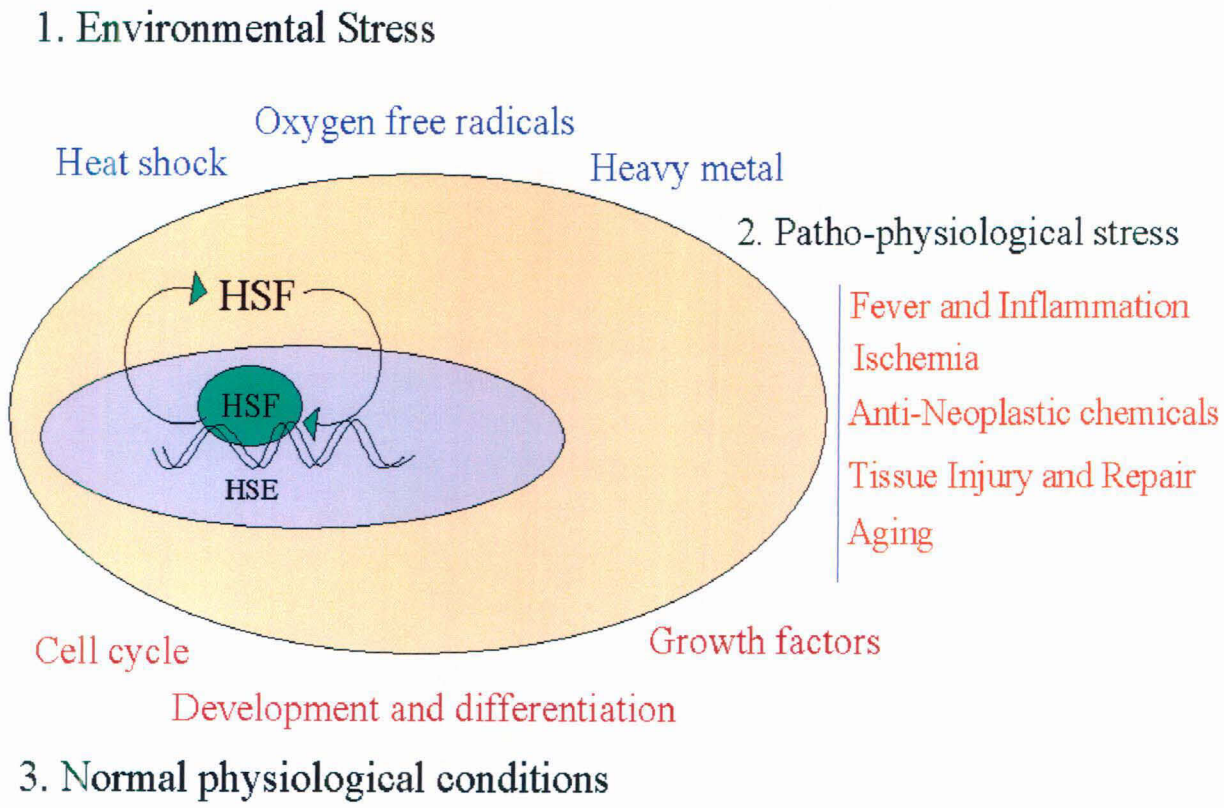


Figure 1.1 Conditions that induce the heat shock response. Various stress lead to activation of HSF, which subsequently bind to HSE, results in elevated expression of HSPs such as HSP70. The regulatory conditions are represented by environmental and physiological stress and normal physiological conditions, including cell growth and development and pathophysiological states. (Morimoto, 1998)

In the recent past many diseases are shown to be associated with protein aggregation, which may arise due to improper folding, mutation and/or imbalance in the protein quality control machinery. Diseases such as Alzheimer's, Parkinson's and prion disease are due to protein aggregation (see review-Muchowski, 2002; Dobson, 2004; Chiti and Dobson, 2006), which results from the alteration of their native structure. Imbalance in the protein quality control machinery leads to cystic fibrosis; where in excessive degradation of mutant CFTR protein (partially unfolded but retains its activity) leading to loss of functional CFTR (Ward *et al.*, 1995; Sharma *et al.*, 2004).Many of the mutant proteins as seen in the case of Huntington's disease lead to protein aggregation, and become resistant to proteasomal degrading machinery.

1.2.2 Mechanism of up-regulation of stress response

Cells employ different strategies to enhance the expression of stress proteins and/or their activation. It is found that under stress condition; the rate of synthesis of mRNA is increased, leading to increase in protein synthesis (Lindquist, 1981; Yost *et al.*, 1990; Morimoto, 1993). Another way of increasing the expression of heat shock proteins is the stabilization of existing mRNA. In the case of HSP70, the mRNA gets stabilized under stress condition, which leads to increased synthesis of HSP70 protein (DiDomenico *et al.*, 1982; Petersen and Lindquist, 1988).

One of the common strategies is activation of existing heat shock proteins by post-translational modifications such as phosphorylation, glycosylation, and chemical modification, which renders resistance to the stress conditions (Sun and MacRae, 2005).

1.2.3 Mechanism of down-regulation of stress response

Under normal conditions, the activities of HSFs remain repressed and localized either in the cytoplasm or in the nucleus in an inert monomeric state (Westwood *et al.*, 1991; Sarge *et al.*, 1993; Wu *et al.*, 1994). These HSFs gets activated upon stress conditions as well as under different physiological conditions such as differentiation (Rallu *et al.*, 1997). Activation of HSFs is influenced by intramolecular interactions with its different domains as well as by the phosphorylation at critical serine residues (Cotto *et al.*, 1996; Kline and Morimoto, 1997). Upon restoration of the normal environmental

condition, the activated heat shock response must be repressed to down regulate the response.

A substantial amount of genetic and biochemical evidence from studies in prokaryotes and eukaryotes supports a role for HSPs in the negative regulation of heat shock response. For example, complexes of HSP70 and HSF trimers have been detected during attenuation of the heat shock transcriptional response (Abravaya *et al.*, 1992; Baler *et al.*, 1992). HSP90 has been shown to have a role in maintaining HSF1 in an inert state (Ali *et al.*, 1998), while HSP70 and HSP40 repress HSF1 by directly binding to its transactivation domain (Mosser *et al.*, 1993; Shi *et al.*, 1995). Heat shock factor binding protein 1 (HSBP1) is another protein that interacts with the hydrophobic heptads of HSF1 and thereby negatively regulates the heat shock response (Satyal *et al.*, 1998). HSBP1 interacts with the trimer of HSF1 and HSP70 but not with the monomeric form of HSF1 (see review- Morimoto, 1998). Although it is known that these molecules transiently bind to HSF1 to repress its activity, molecular details of their binding and consequent repression that occurs remain to be elucidated.

1.3 Classification of Molecular Chaperones

Molecular chaperones are ubiquitously present from prokaryotes to eukaryotes. These heat shock proteins are classified according to their molecular masses into six major families, HSP100, HSP90, HSP70, HSP60, HSP40 and small heat shock proteins family.

1.3.1 HSP100/Clp family

The HSP100 chaperones are members of the large AAA^+ (ATPase Associated with various cellular Activities) superfamily (Schirmer *et al.*, 1996). Proteins in this family form large hexameric structure with unfoldase activity in the presence of ATP (Wickner *et al.*, 1999; Sauer *et al.*, 2004; Inobe *et al.*, 2008). Single or double tiers of ATPase rings form a channel line with key residues for translocation of the unfolded polypeptide chain (Lee *et al.*, 2003). These proteins perform their function by progressively threading the client proteins through a small 16 A^o (1.6 nm) pore, thereby giving each misfolded client protein a second chance to fold (Lee *et al.*, 2003; Lum *et al.*, 2004; Wendler *et al.*, 2007).

The proteins with two tiers of ATPase ring are grouped in to class I chaperones which includes ClpA and ClpB/HSP104 (Sanchez and Lindquist, 1990; Kitagawa *et al.*, 1991; Wickner *et al.*, 1994). ClpB/HSP104 subfamily displays a unique protein disaggregating activity that is used in conjunction with the refolding activities of DnaK/HSP70 (Glover and Lindquist, 1998; Zietkiewicz *et al.*, 2004; Zietkiewicz *et al.*, 2006). Proteins with single tier of ATPase ring are grouped into class II chaperones such as ClpX, whose motor mechanism has been examined in detail (Wojtkowiak *et al.*, 1993).

ClpXP and ClpAP complexes are present in the range of 200 copies per cell, whereas the number of ClpB complexes is 10 times higher. The Clp/HSP100 chaperones can rapidly process their substrates at a rate of 30 molecules per minute and hydrolyzing ATP at rates around 1000 per minute.

1.3.2 HSP90 family

HSP90 is an abundant chaperone in prokaryotes and eukaryotic cytosol, endoplasmic reticulum (ER) and mitochondria. They are essential in late stage of protein folding and are required for the activity of many clients proteins involved in signaling. The prokaryotic homologue of HSP90 "HtpG", is dispensable under non-heat stress condition (Bardwell and Craig, 1987; Bardwell and Craig 1988). HSP90 accounts for approximately 1-2% of the total soluble proteins in unstressed cell indicating its extensive role in normal physiological condition (Lai *et al.*, 1984). HSP90 exists as a homodimer and each subunit is composed of a highly conserved N-terminal (NTD) ATPase domain of ~25 kDa, a charged linker region, middle substrate recognition domain and C-terminal dimerization domain of 12 kDa (Scheibel *et al.*, 1999; Pearl and Prodromou, 2000; Young *et al.*, 2001). In mutlicellular organisms, additional members of HSP90 family such as Grp94 (gp96) are also found in ER, mitochondria and chloroplasts (Mazzarella *et al.*, 1987). These ER-chaperones are known to get up regulated or activated during ER-stress and shown to play a crucial role in maintaining the homeostasis of endoplasmic reticulum (Welihinda *et al.*, 1999). In the unstressed cell, HSP90 is localized in the cytosol bound to HSF1 keeping it in an inert state and upon stress HSP90 dissociates, leaving HSF1 to translocate to the nucleus and trigger stress-induced transcription (Hu and Mivechi, 2003). Upon stress, HSP90 adopts a more general function by reducing its substrate specificity (Freeman and Morimoto, 1996). HSP90 is known to associate with the non-native structures of many proteins and

suppresses the aggregation of a wide range of "client" or "substrate" proteins, thereby functions as a molecular chaperone (Riggs *et al.*, 2004). Its function is regulated by the interaction with several co-chaperones. A recent genomic analysis reveals roles for HSP90 in the secretory pathway, cellular transport, cell cycle and cell division (McClellan *et al.*, 2007).

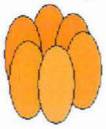
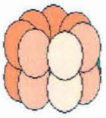
Chaperone family	Examples	ATP	Co-chaperone	Functions
HSP100 (6-7mer) 	ClpB (prok) ClpA HSP104 (euk)	+		- Disaggregation along HSP70 - Proteolysis along with ClpP protease - Thermotolerance
HSP90 (dimer) 	HtpG (pro) HSP90 (euk)	+	Hop, P23, CDC37	- Stress-tolerance - Maintaining stability of steroid hormone receptors, protein kinases
HSP70 (monomer) 	DnaK (pro) HSP70, Hsc70 (euk)	+	DnaJ, GrpE HSP40, HOP, Bag1, HSPBP1	- De novo protein folding - Prevention of aggregation - Solubilization of protein aggregates with HSP100 protein - Regulation of heat shock response - Stabilizing transcription factors and kinases along with HSP90
HSP60 (14/16 mer) 	GroEL (pro) CCT/TriC (euk)	+	GroES prefoldin	- De novo protein folding - Prevention of aggregation - De novo folding of actin, tubulin
sHSPs (8-24 mers) 	IbpA, IbpB (pro) α-crystallin, HSP25 (euk)	–		-Prevention of aggregation -Bind to inclusion bodies -Prevention of aggregation (Possibly several other cellular functions)

Table 1.1 Chaperone families (Mogk *et al.*, 2002)

1.3.3 HSP70 family

The HSP70 family constitutes highly conserved members of the HSP family, found in the cytosol of prokaryotic and eukaryotic organelles such as ER and mitochondria (Craig and Lindquist, 1988; Leustek *et al.*, 1989; Munro and Pehlam, 1986; Alberto *et al.*, 1999). The constitutive form of HSP70 (DnaK in *E.coli*) is called, as Hsc70 (70 kDa heat shock protein and cognate protein) while the inducible form is known as HSP70. They assist a large variety of protein folding processes inside the cell by transiently interacting with their substrates (Gething and Sambrook, 1992). The substrate binding and release cycle is driven by the switching of HSP70 between the ATP binding and hydrolysis process (Szabo *et al.*, 1994). This ATPase cycle is controlled by co-chaperones of the family of J-domain proteins, which targets HSP70 to their substrate and by nucleotide exchange factors, thereby regulating the life-time of the HSP70-substrate complex (Liberek *et al.*, 1991; Misselwitz *et al.*, 1998). These 70-kDa proteins are monomeric with an N-terminal ATPase domain and a C-terminal substrate binding domain (Flaherty *et al.*, 1991; Zhu *et al.*, 1996). A newly synthesized protein binds to HSP70 with its substrate-binding domain, which stimulates the ATPase activity (Slepenkov and Witt, 1998). Further, associating with J-domain proteins such as HSP40/DnaJ leads to speeding of the ATP hydrolysis in the presence of the interacting peptides (Liberek *et al.*, 1991; Pierpaoli *et al.*, 1997). By binding to the partially synthesized peptide sequence, HSP70 prevents them from aggregating. Finally, HSP-organizing protein (HOP) binds to HSP70 and mediates the transfer of peptide from HSP70 for the next step of protein folding releasing HSP70 for the next cycle (Scheufler *et al.*, 2000; Wegele *et al.*, 2004). In eukaryotes, HSP70 homologues are found in the ER (Bip/Grp78) as well as in mitochondria (mtHSP70/Grp75) (Hendershot *et al.*, 1994; Bhattacharyya *et al.*, 1995). Mitochondrial HSP70 is required for the efficient post-translational translocation of precursor proteins into the mitochondrial matrix (Schneider *et al.*, 1994). The endoplasmic reticulum chaperones are shown to be associated with protein transport, protein degradation etc (Gething and Sambrook, 1990).

1.3.4 HSP60 family

The HSP60 families of heat shock proteins are abundant proteins found in all bacteria, mitochondria and plastids of eukaryotic cells. The HSP60 or chaperonin family, including GroEL from bacteria (Georgopoulos *et al.*, 1973), rubisco-binding protein from chloroplasts, HSP60 from mitochondria, and the t-complex polypeptide 1 (TCP-1

complex) from eukaryotic cytosol (Willison *et al.*, 1987), are a group of proteins with distinct ring-shaped, or toroid (double donut) quaternary structure (Ellis and van der Vies, 1991; Roseman *et al.*, 1996; Bukau and Horwich, 1998).

The members of the HSP60 family in *E.coli* (GroEL), mitochondria (HSP60) and chloroplasts (Rubisco) have been shown to mediate the folding of many different proteins *in vivo* and *in vitro.* The non-native polypeptides are captured by binding to hydrophobic sites on an open ring of GroEL. Upon binding of ATP and GroES, major conformational changes occur in GroEL trapping the substrate polypeptide in a hydrophilic chamber for folding (Martin *et al.*, 1991; Fenton *et al.*, 1994; Hartl, 1996; Bukau and Horwich, 1998). Another chaperonin found in the eukaryotic cytosol is CCT (TriC), purified from bovine testis is shown to bind to unfolded proteins, preventing their aggregation and facilitating ATP-dependent refolding of proteins *in vitro* (Gupta, 1990; Gutsche *et al.*, 1999). Unlike most chaperones, CCT is not over-expressed during cell stress.

1.3.5 HSP40 family

This class of proteins mostly functions as co-chaperones together with other chaperones such as HSP70 (Caplan *et al.*, 1993; Qiu *et al.*, 2006). By associating with these chaperones, HSP40 family members enhance the substrate-binding as well as their ATPase activity (Liberek *et al.*, 1991; Pierpaoli *et al.*, 1997; Fan *et al.*, 2003). HSP40 family of molecular chaperones are ATP-independent and characterized by an N-terminus J-domain connected to the substrate-binding C-terminus through a middle linker region (Wawrzynów and Zylicz, 1995; Han and Christen, 2003). It has been shown that the association of HSP70 with Hdj-1/HSP40 is involved in the regulatory mechanism, as over-expression of Hdj-1 prevents the inducible transcription of heat shock genes in the absence of stress (Shi *et al.,* 1998). These chaperone complexes can directly bind to the trans-activation domain of HSF-1, but the mechanism of its repression is not fully elucidated.

1.4 Small Heat Shock Proteins

Within the molecular chaperone family, small heat shock proteins constitute a structurally divergent group and are ubiquitously present in virtually all organisms (Lindquist and Craig, 1988; de Jong WW *et al.,* 1993; Kappe *et al.,* 2002; Haslbeck *et al.,* 2005). Most of the prokaryotes have one or two sHSPs genes (Allen *et al.,* 1992) but higher eukaryotes contain multiple genes e.g. 4 in *D. melanogaster*, 16 in *C. elegans*, 11 in *H. sapiens* (Kappe *et al.,* 2001; Haslbeck *et al.,* 2005; Vos *et al.,* 2008). The reason for this expansion is not clear but it may be due to gene sharing, gene duplication, functional specialization and diversification. The members of the sHSP family vary in length ranging from 143 residues in *C. elegans* to 242 in *Petunia* chloroplast (Lindquist and Craig, 1988; Grimm *et al.,* 1989). All the sHSPs contain about 80-100 residues long conserved region, called the "alpha-crystallin domain" towards the C-terminal region, and is a hallmark feature of this family (Plesofsky-Vig *et al.,* 1992; de Jong *et al.,* 1993; de Jong *et al.,* 1998).

Although proteins belonging to the small heat shock protein family are diverse in sequence and size, most share characteristic features, including monomeric molecular mass in the range of 12-43 kDa, formation of oligomers with dynamic quaternary structure and chaperone-like activity in preventing aggregation of proteins (Lindquist and Craig, 1988; van den Oetelaar *et al.,* 1990; Kato *et al.,* 1992; Horwitz, 1992; Kato *et al.,* 1993b; Jakob *et al.*, 1993; Merck *et al.*, 1993). It has been shown that sHSPs undergo structural alteration under stress conditions and expose their hydrophobic surfaces (Raman and Rao, 1994; Raman *et al.*, 1995). Structural transition seems to be essential and biologically relevant process as non-native proteins transiently bind with the exposed hydrophobic patches (Raman and Rao, 1994; van Montfort *et al.*, 2001). Further, it was demonstrated that heat stress, chemical stress, oxidative stress etc lead to increase in their chaperone activity and subunit exchange (Gu *et al.*, 2002; Bova *et al.*, 2002). Structural alteration combined with increased chaperone activity is the general mechanism of its action under stress conditions (Datta and Rao, 2000). Besides their role in preventing protein aggregation *in vitro*, sHSPs have been shown to perform many cellular functions under normal as well as stress conditions (Ciocca *et al.,* 1993; Klemenz *et al.,* 1993; Arrigo, 2000; Kitagawa *et al.,* 2000). sHSPs are shown to undergo post-translational modifications such as phosphorylation, disulfide linkage during various cellular processes, which play a crucial role in its *in vivo* activity (Lavoie *et al.*, 1993; Groenen *et al.*, 1994; Kato *et al.*, 2002). Many reports suggest that phosphorylation leads to increased subunit exchange and higher chaperone activity under stress

conditions. It appears that reduction in the oligomer size and structural transitions is generally associated with the mechanism of action of sHSPs in preventing the protein aggregation under stress condition (Ito *et al.*, 2001b). The human genome encodes for eleven small heat shock protein namely HSPB1 (HSP27), HSPB2 (MKBP), HSPB3, HSPB4 (αA-crystallin), HSPB5 (αB-crystallin), HSPB6 (HSP20), HSPB7 (cvHSP), HSPB8 (HSP22), HSPB9, HSPB10 (ODF1), HSPB11 (HSP16.5) (Kappe *et al.*, 2001; Vos *et al.*, 2008). The expression of these sHSPs varies from tissue type and in a stress-dependent manner. Most of these sHSPs are present in muscle tissue, brain, kidney, liver, heart and eye lens (Welsh and Gaestel, 1998). In muscle cell, as many as six out of eleven sHSPs are present and perform different function during stress as well as in differentiation process (Sugiyama *et al.*, 2000).

Mammalian sHSPs	Chromosomal localization	Tissue distribution	Sub-cellular localization	Associated Diseases
HSPB1 (HSP27)	7q11.23	Ubiquitous	Cytosol	Charcot-Marie-Tooth disease
HSPB2 (MKBP)	11q23-23	Muscle	Cytosol/mito.	Unknown
HSPB3	5q11.2	Muscle	Unknown	Unknown
HSPB4 (αA-crystallin)	21q22.3	Eye lens	Cytoplasm	Cataract
HSPB5 (αB-crystallin)	11q22.3-q23.1	Ubiquitous	Cytoplasm	Cataract, DRM
HSPB6 (HSP20)	19q13.12	Muscle & Brain	Cytosol	Unknown
HSPB7 (cvHSP)	1p36.23-p34.3	Muscle	Nucleus/Cyto	Muscular dystrophy
HSPB8 (HSP22)	12q24.23	Muscle & Brain	Cytoplasm, plasma mem.	Distal hereditary motor Neuropathy, CMT disease
HSPB9	17q21.2	Testis	Cytosol	In certain tumors
HSPB10 (ODF)	8q22.3	Testis	Sperm cell tails	Unknown
HSPB11 (HSP16.2)	1p32.1-p33	Unknown	Cytosol	In certain tumors

Table 1.2 Lists of mammalian sHSPs and its associated diseases (Vos *et al.*, 2008)

Among all the mammalian sHSPs known so far only four of them HSP27, αA-crystallin, αB-crystallin and HSP22 are well characterized and studied in detail. The heat or stress inducibility has been shown for only three of the sHSPs, which includes HSP27, αB-crystallin and HSP22 (Kato *et al.*, 1993b; Kato *et al.*, 2002; Chowdary *et al.*, 2004). HSP27 and αB-crystallin are shown to have a role in the prevention of apoptosis induced by various agents such as TNF-α, UV, doxorubicin, H_2O_2, and staurosporine (Mehlen *et al.*, 1995; Bellyei *et al.*, 2007, Turakhia *et al.*, 2007; Laskowska, 2007). Both, HSP27 and αB-crystallin are shown to interact with pro-caspases and inhibit their maturation process (Garrido *et al.*, 1999; Kamradt *et al.*, 2001; Voss *et al.*, 2007). αB-crystallin also interacts with Bax; a pro-apoptotic protein, this interaction might inhibit Bax translocation to mitochondria, thereby help in preventing apoptosis (Mao *et al.*, 2004). Besides their role in protection, sHSPs also help in the maturation or degradation of non-native targets via the ubiquitination-mediated proteasomal degradation pathway (den Engelsman *et al.*, 2003; Parcellier *et al.*, 2003; Haslbeck *et al.*, 2005). During the muscle differentiation process, HSP27 level gets up regulated by 3-fold indicating a possible role in the differentiation process (Ito *et al.*, 2001a). HSP25/27 has been found to be necessary for the functional differentiation of P19 into cardiomyocytes (Stahl *et al.*, 1992; Davidson and Morange, 2000). HSP27 is shown to be involved in the differentiation process of other cell lines such as B lymphoma cells, keratinocytes and chrondrocytes (Favet *et al.*, 2001; Duverger *et al.*, 2004). HSP27 up-regulation during differentiation is correlated with an increase in phosphorylation and in a shift of HSP27 oligomers toward high molecular masses (Mehlen and Arrigo, 1994; Chaufour *et al.*, 1996; Chiesa *et al.*, 1997). One of the functions of sHSPs is their ability to interact with several cytoskeletal as well as nucleo-skeletal elements such as, lamins and actin filaments (Verschuure *et al.*, 2002; Adhikari *et al.*, 2004). The interaction of HSP25 with actin microfilaments has been shown to provide stability and regulates its dynamics (Benndorf *et al.*, 1994; Bryantsev *et al.*, 2002). The role of sHSPs such as HSP27 has also been documented in regulation of various transcription factors including NF-kB, a ubiquitous transcription factor (Parcellier *et al.*, 2003; Park *et al.*, 2003). HSPB2, also known as myotonic dystrophy protein kinase binding protein (MKBP), interacts with the myotonic dystrophy protein kinase (DMPK) and enhances its kinase activity (Iwaki *et al.*, 1997; Suzuki *et al.*, 1998). HSPB2 knock out studies show that the mice develop abnormalities in skeletal muscle development (Morrison *et al.*, 2004). Most of the sHSPs such as HSPB3 and HSPB6 (HSP20) are restricted to muscle tissues such as heart and skeletal muscle (Verschuure *et al.*, 2003). However, their exact localization and function under normal as well as stress condition are not yet clear. HSPB3 forms complexes with HSPB2 during the muscle differentiation

process; however the functional significance of this interaction is not clearly understood (Sugiyama *et al.*, 2000). HSPB6 (HSP20) is expressed in smooth muscles and appears to play a role in muscle relaxation and cardio-protection. HSPB7 (cvHSP) is expressed in heart and skeletal muscle, the expression is enhanced in aging muscle reflecting a cellular adaptation to higher proteotoxic stress conditions (Krief *et al.*, 1999; Doran *et al.*, 2007).

Two members of the sHSPs family, HSPB9 and HSPB10 are exclusively expressed in testis (Fontaine *et al.*, 2003). These are not well characterized yet, except for their association with cytoskeletal or associated proteins such as DynLT1 (a light chain component of dyein) (de Wit *et al.*, 2004). The recently reported HSPB11 (HSP16.2) was shown to form oligomeric complexes and prevent the aggregation of *in vitro* denaturated aldolase and glyceraldehyde-3-phosphate dehydrogenase in accordance with the chaperone model of HSPB1 and HSPB5. HSPB11 over-expression protected cells against etoposide-induced cell death which correlated with a decreased level of release of mitochondrial cytochrome *c* into the cytosol. Inhibiting HSP90 function completely abrogated the protective effect of HSPB11 (Bellyei *et al.*, 2007).

Many of the sHSPs are known to be associated with neurological and muscle-related diseases. The expressions of many of sHSPs such as HSP27 are enhanced in cancers and neurological disorders and are localized to degenerating neurons (Sun and MacRae, 2005).

1.4.1 Alpha-crystallin

α-crystallin accounts for more than ~ 50 % of the lens fiber cells' protein and was originally considered as a structural protein (Bloemendal, 1981; Wistow and Piatigorsky, 1988). It is the major water-soluble, multimeric protein of the eye lens made up of two distinct gene products, *αA-crystallin* and *αB-crystallin* (Wistow and Piatigorsky, 1988; de Jong *et al.*, 1993). Heat shock proteins, first discovered in heat-induced puffs of polytene genes of *D. melanogaster*, showed sequence homology to α-crystallin (Tissieres *et al.*, 1974; Ingolia and Craig, 1982). Subsequently heat-induced expression of α-crystallin was demonstrated, identifying it to be a heat shock protein (Klemenz *et al.*, 1991). In recent years, it has been generally accepted that α-crystallin is an ATP-independent molecular chaperone, which binds to partially denatured proteins to prevent their aggregation (Rao *et al.*, 1993; Raman and Rao, 1994; Raman and Rao, 1997;

Muchowski and Clark, 1998). It was shown that α-crystallin has chaperone-like properties and effectively binds to partially denatured proteins; thereby suppressing non-specific irreversible aggregation and keeping them in the folding competent state (Raman and Rao, 1994; Raman and Rao, 1997; Rao *et al.,* 1998). Lens, where the protein concentration is ~ 400 mg/ml (Fagerholm *et al.*, 1981), needs these chaperones to prevent aggregation induced by UV-light, heat, age-related post translational modifications etc.

1.4.2 αA-Crystallin

αA-Crystallin, a member of small heat shock protein family was originally identified as a hetero-oligomer with αB-crystallin from eye lens (Siezen *et al.,* 1978). αA-crystallin gene is localized on chromosome 21 of human that codes for 173 amino acid residues long polypeptide (Thompson *et al.,* 1987; Horwitz, 2003). It shows ~ 58% and ~ 45% sequence similarity with αB-crystallin and other sHSPs respectively (de Jong *et al.,* 1993). The expression of αA-crystallin is largely restricted to the lens, but it is also found in thymus, spleen and liver (Kato *et al.,* 1991). It is localized in the cytoplasmic region and the localization changes in response to stress conditions (Mackay *et al.,* 2003). Functionally, αA-crystallin is primarily involved in the prevention of aggregation of target proteins induced due to variety of insults such as heat, chemical, osmotic and light (Raman and Rao, 1994; Borkman *et al.,* 1996; Raman and Rao, 1997). The peptides of αA-crystallin spanning from 70-88 residues, suppress amyloid fibril formation of Abeta protein (Ecroyd and Carver, 2008). It is also involved in the stabilization of cytoskeletal elements such as actin and prevents its depolymerization (Wang and Spector, 1996). αA-crystallin knockout mice develops congenital cataract as a result of the presence of inclusion bodies in the lens fiber cells and have reduced ocular lens size (Brady *et al.,* 1997). The involvement of αA-crystallin in the prevention of apoptosis induced by staurosporine, UV-treatment etc has been reported (Mackay *et al.,* 2003). Upon straurosporine treatment, it interacts with Bcl-Xs and Bax, thereby sequestering them from translocation to the mitochondria, while, in UVA-treated cells, the prevention of apoptosis is achieved by activating the Akt (the survival pathway) pathway (Mao *et al.,* 2004; Liu *et al.,* 2004). During cell division, the level of αA-crystallin gets modulated and it has been shown that mice lacking αA-crystallin (αA -/-) exhibit slower growth (Bai *et al.,* 2004). Mutations in αA-crystallin R116C, R49C are known to cause dominant congenital cataract while G98R mutation leads to presenile cataract (Litt *et al.,* 1998;

Mackay *et al.,* 2003; Santhiya *et al.,* 2006). The G98R mutant exhibits folding defects and is prone to aggregation (Singh *et al.,* 2006). The R116C mutant of αA-crystallin shows significantly less binding to actin compared to wild type αA-crystallin (Brown *et al.,* 2007). Further, the R116C mutant shows less chaperone-like activity, whereas the chaperone-like activity of R49C mutant remains unaltered (Kumar *et al.,* 1999; Mackay *et al.,* 2003). The disease phenotype appears to be due to the mis-localization of the mutant (R49C) protein to the nucleus under normal condition compared to the cytoplasmic localization of the wild type protein (Mackay *et al.,* 2003). αA-crystallin undergoes phosphorylation in the C-terminal region (serine 122) by the cAMP-dependent pathway (Chiesa *et al.,* 1987). The other phosphorylation sites present in αA-crystallin are not clearly known; it appears that there are three phosphorylation sites between 122 and 173 residues, which are phosphorylated by its autokinase activity (Kantorow and Piatigorsky, 1998). Although αA-crystallin is seen in non-lenticular tissues its functions in those tissues is not known.

1.4.3 αB-crystallin

αB-Crystallin is one of prominent members of the human small heat shock protein family (Klemenz *et al.,* 1991). αB-crystallin, a member of the crystallin family which includes α-, β- and γ-crystallin, was best known for its refractive and structural properties in the vertebrate lens (Harding and Dilley, 1976). αB-crystallin was included in the small heat shock protein family when Ingolia and Craig (1982) reported that α-crystallin (αA- and αB-crystallin) have high homology with the members of the small HSP genes in *D. melanogaster.* Later, Klemenz *et al.,* (1991) showed that the expression of αB-crystallin is induced by heat shock and also by other stress conditions such as osmotic shock, arsenite and hypertonic conditions (Kato *et al.,* 1993b). The gene for αB-crystallin is located in the human chromosome number 11 and it codes for 175 amino acid residues. Promoter analysis showed that it has canonical HSE elements and is stress-inducible (Somasundaram and Bhat, 2000). αB-crystallin was initially thought to be restricted to the lens and serve a structural function in the lens. However, later it was found to be ubiquitously present in many non-lenticular tissues such as skeletal muscle, cardiac muscle and brain (Bhat and Nagineni, 1989). αB-crystallin is a good example of gene sharing, which means that the same gene acquires an additional function without duplication. In the lens it co-exists with αA-crystallin as a hetero-oligomer and helps in

the maintenance of lens transparency (Siezen *et al.,* 1978; Bloemendal, 1982). The presence of αB-crystallin in many different tissues is indicative of its diverse functions. In these tissues it exists either as a homo-oligomer or as a hetero-oligomer with other sHSPs such as HSP27, HSP22, and HSP20 (Sugiyama *et al.,* 2000). It has monomer molecular mass of 20 kDa but exists as a large oligomer of ~ 600-800 kDa (Spector *et al.,* 1971; Ito *et al.,* 2001b). The structure of αB-crystallin is thought to have three domains - the N-terminal hydrophobic domain, the conserved C-terminal "α-crystallin domain" and an exposed, flexible C-terminal extension (de Jong *et al.,* 1993). Although the crystal structure of αB-crystallin is not available due to its polydisperse nature, other structural techniques such as cryo-electron microscopy and 3D-image reconstruction show that αB-cystallin is spherical and consists of ~ 32 subunits, 18 nm in diameter and a hollow core with ~ 8 nm in the central cavity (Figure 1.4.3.1) (Haley *et al.,* 1998). αB-crystallin is shown to have predominantly β-sheet structure with about 5% α-helical structure (Li and Spector, 1974; Siezen and Argos, 1983); however, modifications such as oxidation leads to increase in random coil with subsequent decrease in the β-sheet or α-helix content.

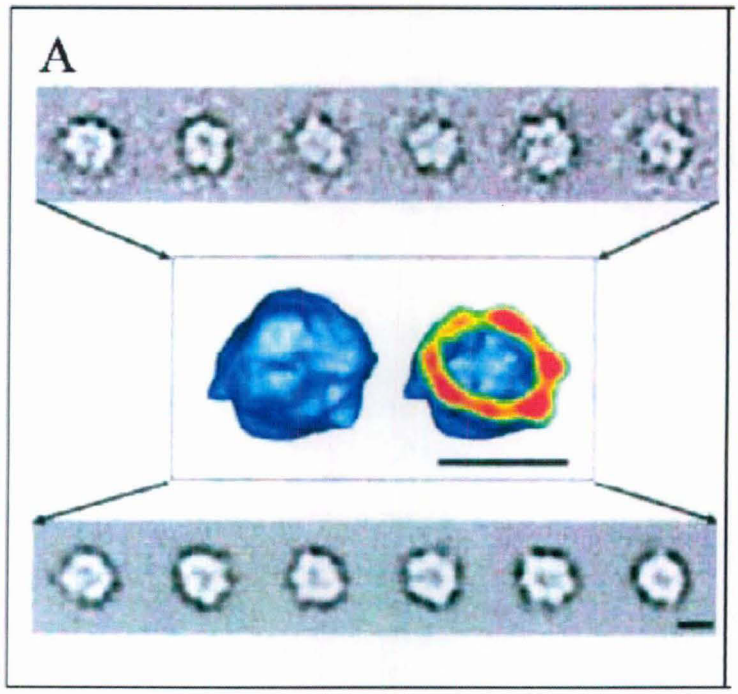


Figure 1.4.3.1 Cryoelectron microscopy structure of α-crystallin (Haley *et al.,* 1998)

αB-Crystallin acts as a molecular chaperone and prevents the stress-induced aggregation of target proteins *in vitro* (Horwitz, 1992). Studies from our laboratory have shown that it undergoes structural transitions at higher temperature thereby exposing or reorganizing the hydrophobic patches to which target proteins bind (Raman and Rao, 1994). αB-crystallin exhibits very little chaperone like activity at room temperature, which increases significantly at higher temperature (Raman and Rao, 1994). Studies from our laboratory with diverse model systems including photo-aggregation of α-crystallin, DTT-induced aggregation of insulin and thermal aggregation of β- and α-crystallin show that α-crystallin undergoes quaternary as well as tertiary structural changes (Rao *et al.,* 1993; Raman and Rao, 1994; Raman *et al.,* 1995; Raman and Rao, 1997). It is shown that tertiary structural changes precede quaternary structural changes. Temperature-induced enhancement of chaperone-like activity appears to be a general phenomenon since it needs to function at non-permissible temperatures. It is found that the induced alterations in the structural parameters are responsible for the enhanced chaperone activity which is further corroborated by the fact that the agents which bring about structural changes also lead to enhanced activity. For example, structural changes induced by low concentration of urea and GdmCl result in enhanced chaperone activity (Raman and Rao, 1994; Das and Liang, 1997). Small molecules including arg-HCL, pantethine, ATP etc lead to subtle changes in tertiary structure, leading to increase in the chaperone activity (Clark and Huang, 1996; Srinivas *et al.,* 2003). Studies from our laboratory have demonstrated that swapping the N-terminal domain and the C-terminal extension of αA- and αB-crystallin results in higher chaperone activity (Kumar and Rao, 2000; Pasta *et al.,* 2002). The N-terminal region SRLFDQFFG is involved in oligomer assembly while the IXI/V motif in the C-terminal region is shown have role in subunit interactions and stability (Pasta *et al.,* 2003; Pasta *et al.,* 2004). αB-crystallin also prevents or suppresses aggregation induced by heat, UV-light, metal ions, chemicals, osmotic stress, oxidation etc (Horwitz, 1992; Rao *et al.,* 1993; Wang and Spector, 1995; Borkman *et al.,* 1996). It is now generally accepted that αB-crystallin is an ATP-independent molecular chaperone but shows autokinase activity (Kantorow and Piatigorsky, 1998). However, it is found that presence of ATP significantly enhances the reactivation yield of citrate synthase (Muchowski *et al.,* 1998). Equilibrium dialysis, 31^{P}-NMR and fluorescence studies suggest that α-crystallin binds to ATP in a temperature-dependent manner, but it remains non-hydrolyzed (Palmisano *et al.,* 1995).

The functionality of αB-crystallin is modulated by post-translational modifications such as deamidation and phosphorylation (Sun and MacRae, 2005). Post-translational

modification of α-crystallin during aging is believed to be responsible for its acidic forms; however it became clear later that the acidic form is due to phosphorylation at specific residues and not because of deamidation at Asn and Gln residues (Palmer *et al.,* 1969; van Kleef *et al.,* 1976; Bloemendal, 1977). The phosphorylation of αB-crystallin plays a crucial role in modulating its function and chaperone-like activity. In the bovine lens approximately 30% of α-crystallin remains phosphorylated. Studies using mass spectrometry and radioactive labeling techniques demonstrated that αB-crystallin gets phosphorylated at three serine residues: Ser-19, Ser-45 and Ser-59 (Cheisa *et al.,* 1987; Smith *et al.,* 1992). The phosphorylation of αB-crystallin at Ser-19 is specific during mitotic process and also found to be age-dependent (Kato *et al.,* 1998). On the other hand, αB-crystallin gets differentially phosphorylated at Ser-45 and Ser-59 residues and is dependent upon stress condition. From the specificity of phosphorylation of α-crystallin, it appears that there may be at least two different kinases responsible for their phosphorylation.

The signaling molecules MKK3 and MKK6 activate p38-MAP kinase, which in turn activates MAPKAP-2/3 (Raingeaud *et al.,* 1996). During stress conditions such as ischemia, heat stress, and chemical stress, MAP kinases get activated, which in turn results in phosphorylation at different serine residues of αB-crystallin (Zu *et al.,* 1997; Ito *et al.,* 1997; Nguyen and Shiozaki, 1999).Various studies show that p44/p42 MAP kinase is responsible for the phosphorylation of αB-crystallin at Ser-45 residue, whereas p38-MAP kinase activated MAPKAP-2/3 leads to phosphorylation at Ser-59 residue (Kato *et al.,* 1998). Studies from our laboratory have shown that the phosphorylated αB-crystallin dissociates to smaller oligomer and has higher chaperone activity (Ahmad *et al.,* 2008). Although, the correlation between chaperone activity and oligomer size is not well established, it is generally believed that smaller oligomers have higher activity. Phosphorylation of αB-crystallin affects its functionality both in normal and disease conditions. It has been shown that phosphorylation of Ser-59 is necessary and sufficient for the prevention of ischemia-induced apoptosis in cardiomyocytes (Morrison *et al.,* 2003). αB-Crystallin gets phosphorylated at Ser-45 and Ser-59 upon exposure of cells to PMA, okadaic acid, arsenite, NaCl and H_2O_2 (Ito *et al.,* 1997). Inhibiting the p38-MAP kinase by specific inhibitors such as SB202190 (p38-MAP kinase inhibitor) leads to inhibition of phosphorylation induced by arsenite, NaCl, and anisomycin while PD098059 (p44/42-MAP kinase inhibitor) inhibits phosphorylation induced by PMA, okadaic acid (Ito *et al.,* 1997). During cell division, Ser-45 phosphorylated αB-crystallin is localized to the cytoplasm while αB-crystallin phosphorylated at Ser-59 position gets localized to

centrosome and mid bodies (Kato *et al.,* 1998; Inaguma *et al.,* 2001). Lens epithelial cells derived from mice lacking αB-crystallin are shown to have genomic instability and hyperproliferation (Bai *et al.,* 2003). Recently, it has been demonstrated that αB-crystallin is essential for the degradation of cyclin D1, an essential component of G1 phase of cell cycle (Lin *et al.,* 2006). The importance of αB-crystallin is further corroborated by the fact that it is a part of cell cycle dependent golgi reorganization and interacts with intermediate filaments during mitosis (Djabali *et al.,* 1999; Gangalum *et al.,* 2004). The interaction of αB-crystallin with intermediate filaments has been demonstrated during normal conditions, which increases further with increasing temperature, serum starvation and hypertonic stress (Djabali *et al.,* 1997). In cardiomyocytes, αB-crystallin is distributed in the central region of the I-bands (Z lines) together with desmin filaments during ischemia (Golenhofen *et al.,* 1998; Bennardini *et al.,* 1992). The translocation of αB-crystallin to the Z lines of myocardium is shown to be phosphorylation-dependent. In addition to intermediate filaments, αB-crystallin also interacts with other cytoskeletal elements such tubulins and actin microfilaments. In conditions of proteasomal inhibition, αB-crystallin translocates to the actin cytoskeleton (Verschuure *et al.,* 2002). Importantly, αB-crystallin-coated MAP microtubule resists nocodazole and calcium-induced disassembley, suggesting that it provides resistance to microtubules from disassembly (Fujita *et al.,* 2004). Recently, it has been shown from our laboratory that in myoblasts, αB-crystallin migrates to the nucleus upon heat stress, where it colocalizes with SC-35 and lamin A/C (Adhikari *et al.,* 2004). The migration of αB-crystallin to the nucleus is of interest, as it does not contain NLS sequence. It has been shown that αB-crystallin interacts with SMN-like proteins, which might be involved in carrying αB-crystallin to the nucleus (den Engelsman *et al.,* 2005). In addition to its migration to the nucleus, cytoplasmic redistribution of αB-crystallin has also been documented under normal as well as stress conditions. During ischemic condition, it translocates to the mitochondria in a phosphorylation-dependent manner (Jin *et al.,* 2008). Further, it was found that mitochondria incubated with a recombinant mutant form of αB-crystallin that mimics Ser-59-phospho-αB-crystallin exhibited decreased calcium-induced MPT pore opening. These results indicate that mitochondria may be among the key components in stressed cells with which αB-crystallin interacts and modulates MPT pore opening thus, reducing Ischemia/Reperfusion (IR) injury (Jin *et al.,* 2008). α-Crystallins restrict release of cytochrome-C, repress activation of caspase-3 and block degradation of PARP thus preventing apoptosis induced by staurosporine, etoposide etc (Mao *et al.,* 2004). Upon TNF-α treatment, αB-crystallin inhibits the activation of caspase-3 by interacting with pro-

active caspase-3 (p24) (Kamradt *et al.,* 2001). It has been shown that Ser-59 phosphorylation is essential and sufficient for its anti-apoptotic activity. During the muscle differentiation process the level of αB-crystallin goes up by 10-fold and shown to have anti-apoptotic ability by preventing the activation of caspase-3 (Ito *et al.,* 2001a; Kamradt *et al.,* 2002). The up-regulation and anti-apoptotic behavior of αB-crystallin during differentiation is of importance as none of the anti-apoptotic proteins are shown to be up-regulated during this process. In the skeletal muscle, αB-crystallin promoter has muscle-specific enhancer elements in the region –427 to –259, suggesting the importance of αB-crystallin in muscle (Dubin *et al.,* 1991). Knock out mice lacking αB-crystallin have lower muscle mass. In addition, a point mutation, R120G in αB-crystallin leads to desmin related myopathy (DRM), implying its role in muscle maintenance and homeostasis (Wang *et al.,* 2001). A missense mutation of CRYAB, Arg157His, was found in a familial dilated cardiomyopathy (DCM) patient and the mutation was found to decrease the binding to titin/connectin heart-specific N2B domain without affecting distribution of the mutant crystallin protein in cardiomyocytes (Inagaki *et al.,* 2006). Recently, the involvement of αB-crystallin has been documented in cancerous conditions. The expression profile of αB-crystallin appears to be tumor type and stage specific (Gruvberger-Saal and Parsons, 2006; Sitterding *et al.,* 2008).

From the foregoing description, it is clear that in addition to helping in maintaining the structural integrity of the target proteins and preventing their aggregation, αB-crystallin has several critical cellular roles to play.

1.5 Scope of the present study

Understanding the *in vivo* functions of αB-crystallin, a member of small heat shock proteins, is important due to its role in normal physiological activities such as cell division, differentiation, cytoskeletal dynamics, apoptosis and its possible role in several disease conditions such as cataract, myopathies, neurodegenerative diseases, ischemia and cancer. Under *in vitro* condition, αB-crystallin is shown to interact with filamentous actin but not with monomeric actin; however its *in vivo* association with actin is not known. Importantly, under stress conditions such as ischemia and hyperthermia filamentous actin tends to dissociate into monomers. The interaction of αB-crystallin with actin *in vivo*, particularly in the context of stress needs to be investigated. In the heart as well as in skeletal muscle, αB-crystallin has been shown to play an important role in

preventing apoptosis and maintaining muscle homeostasis. In the heart it has been shown that disintegration of actin microfilaments leads to apoptosis and is major cause of myocardial infarction.

We set out to investigate the role of αB-crystallin in cytoskeletal dynamics and in myoblast differentiation. We have used H9C2 cardiomyoblasts as a model system for investigating *in vivo* interactions of αB-crystallin with cytoskeletal system and C2C12, mouse myoblasts for its role in differentiation. The modulation of cytoskeletal architecture is associated with differentiation process as well. During muscle differentiation, the level of αB-crystallin increases by 10-fold and shown to have anti-apoptotic function during this process. We have made αB-crystallin stably expressing C2C12 myoblasts, C2C12-CRYAB, to investigate the role of αB-crystallin in differentiation process. We have also used functionally compromised, myopathy causing mutant, R120G for comparative studies. Results show specific phosphorylation dependent role for αB-crystallin in cytoskeletal stability and dynamics. Studies with C2C12-CRYAB show significant delay in differentiation process due to delayed exit from cell cycle and alterations in the expression of differentiation regulators such as MyoD, myogenin etc. Studies with disease causing mutant, R120G show increased cell death possibly connected with muscle loss in cardiomyopathies.

CHAPTER 2

Association of αB-crystallin with Filamentous actin

2.1 Introduction

Cells respond to environmental stress by preferential synthesis and accumulation of a family of proteins called as heat shock proteins (HSPs), which assist them to survive under these unfavorable conditions (Landry *et al.*, 1982; Li and Werb, 1982; Parsell and Lindquist, 1993). Small HSPs (sHSPs), a subset of the HSP family, have been shown to be involved in many cellular processes that help in the survival of cells under conditions of stress (Kato *et al.*, 1993c; Andley *et al.*, 2000; Mao *et al.*, 2004). Inspite of the increasing number of reports, the exact mechanisms by which sHSPs confer stress protection to cells are not clearly understood. Stresses such as ischemia and hyperthermia lead to enhanced expression of αB-crystallin, a member of the sHSP family, implying its importance in cell survival (Chiesi *et al.*, 1990; Klemenz *et al.*, 1991; Kato *et al.*, 1993b). αB-crystallin is shown to act as a molecular chaperone and prevent the stress-induced aggregation of target proteins *in vitro* (Horwitz, 1992; Rao *et al.*, 1993; Wang and Spector, 1995). Earlier studies from our laboratory have shown that the chaperone-like activity of α-crystallin in preventing aggregation of target proteins increases several fold at heat shock temperature (Raman and Rao, 1994; Raman *et al.*, 1995). In addition to its chaperone functions *in vitro*, the role of αB-crystallin is well documented in many cellular events such as cell division, differentiation and apoptosis (Kato *et al.*, 1998; Ito *et al.*, 2001a; Bai *et al.*, 2004; Kamradt *et al.*, 2005).

Both ischemia and heat-stress cause extensive damage to the cytoskeleton that includes collapse of the intermediate filament network, disruption of the microtubules, rearrangement and/or disorganization of actin microfilaments (Glass *et al.*, 1985; Welch and Suhan, 1985; Ganote and Vander Heide, 1987). It has also been observed that a brief period of hyperthermia is associated with enhanced post-ischemic ventricular recovery in rats (Currie *et al.*, 1988). Interestingly, cardiac ischemia results in rapid translocation of αB-crystallin from cytosolic pool to intercalated discs and Z-lines of the myofibrils (Golenhofen *et al.*, 1998), implicating its critical role in the protection of myocardium. A point mutation (R120G) in αB-crystallin leads to its abnormal interaction with desmin and causes desmin-related myopathies in the heart tissue (Vicart *et al.*, 1998; Wang *et al.*, 2001). Studies from our laboratory as well as those of others have shown that reduced chaperone activity of R120G-αB-crystallin, which might be the molecular basis for the observed pathology (Kumar *et al.*, 1999; Bova *et al.*, 1999). Reducing αB-crystallin gene expression using anti-sense

strategy leads to disruption of the actin microfilament network (Iwaki *et al.,* 1994), further supporting the important role played by this protein in the maintenance of microfilament integrity and cellular survival. In addition to its regulation of microfilament stability, αB-crystallin has also been shown to interact with microtubules and proteins of the intermediate filament family and confer stability during conditions of stress (Perng *et al.,* 1999; Fujita *et al.,* 2004).

Disruption of the cytoskeleton and disaggregation of actin fibers are among the most immediate effects of stress such as heat shock (Glass *et al.,* 1985; Welch and Suhan, 1985). Stabilization of actin fibers under stress conditions, therefore, is one of the critical requirements for survival. Moreover, several cellular processes, such as motility and pinocytosis, involve extensive actin polymerization-depolymerization (Theriot and Mitchison, 1991; Nakase *et al.,* 2004). αB-crystallin has been reported to interact with actin *in vitro* and this interaction increases with increasing temperature (Bennardini *et al.,* 1992). However, the molecular mechanisms of interaction of αB-crystallin with actin filaments *in vivo* are not clearly understood.

This chapter describes our investigations on the role of αB-crystallin in stress dependent alterations in actin dynamics. Since αB-crystallin is shown to have cardio-protective role, we have chosen H9C2 rat cardiomyoblast cell line for our study. We find that αB-crystallin associate with actin stress fibers upon heat stress. We have also investigated the pinocytosis process, to probe the functional consequences of heat-induced alteration in the actin polymerization-depolymerization process. Results provide interesting insight into the interaction of αB-crystallin with filamentous actin *in vivo*. Our study suggests that αB-crystallin has a major role in protecting cells from stress-induced damage and in the preservation of cellular architecture.

2.2 Materials and Methods

2.2.1 Antibodies and Reagents

Rabbit polyclonal antibodies for αB-crystallin, phosphoserine-59-αB-crystallin and phosphoserine-45-αB-crystallin were obtained from Stressgen Biotechnologies, Canada; monoclonal anti-HA antibodies and protease inhibitor cocktail were procured from Roche Applied Sciences, USA and FITC-dextran, Rhodamine-phalloidin conjugates, Alexa-488-conjugated secondary antibodies were obtained from Molecular Probes, Oregon, USA. DMEM, fetal calf serum (FCS) and cytochalasin B was purchased from Sigma Chemical Company, U.S.A. Lipofectamine-2000 was procured from Invitrogen, U.S.A. HRPO-conjugated anti rabbit secondary antibodies and Enhanced Chemiluminescence (ECL) western blot detection kit were obtained from Amersham Biosciences, U.S.A. Vectashield mounting medium containing DAPI was purchased from Vector Laboratories, USA. Inhibitors of p44-MAP kinase (PD098059) and p38-MAP kinase (SB202190) were obtained from Calbiochem, EMB Biosciences Inc., Germany.

2.2.2 Cell Culture and Heat Treatment

H9C2, rat myoblast cell line was maintained at sub-confluent densities in DMEM supplemented with 10% FCS, penicillin (100 units/ml) and streptomycin (100 μg/ml) at 37 °C in a humidified chamber containing 5% CO_2. Cells grown in T75 flasks were incubated at 43 °C in a water bath for different time points as indicated. Cells were lysed in 50 mM Tris-Cl, pH 7.4, containing 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 0.2 mM Na_3VO_4, 0.2% NP-40 and protease inhibitor cocktail (lysis buffer). For immunoflourescence studies, H9C2 cells grown on cover slips were subjected to heat shock at 43 °C for different time intervals as indicated. For recovery, H9C2 cells subjected to heat stress for 60 min were incubated at 37 °C, in a humidified CO_2 incubator for different length of time. Cells were fixed with 3.7% formaldehyde after each time point and processed for confocal analysis.

Primary cultures of neonatal rat ventricular myocytes (NRVMs) were prepared from 1–4-day-old Harlan Sprague-Dawley rats as described (Zechner *et al.,* 1997). Briefly, hearts were dissected in DMEM/air; the apical two-thirds of the ventricles were dissected away

from the atria. After mincing and washing the ventricles twice with air-compatible DMEM, cells were isolated by multiple rounds of 10-min-long tissue dissociation with 0.001% trypsin. After each incubation with trypsin, the supernatant was added to an equal volume of DMEM/F-12 (1:1) containing 20% fetal bovine serum, and all of the supernatants were combined. The cell suspension was filtered through 40 μm gauge filters and plated on to collagen coated petridishes or gelatin coated coverslips. NRVMs, grown on cover slips were subjected to heat shock at 43 °C for 1 hr. For recovery, heat-stressed myocytes were incubated at 37 °C, in a humidified CO_2 incubator indicated time period. Cells were fixed with 3.7% formaldehyde after each time point and processed for confocal analysis.

2.2.3 Plasmid and Transient Transfection

The plasmid pCDNA 3.1HA-3A αB-crystallin was a kind gift from Dr. C. Glembotski (Morrison *et al.,* 2003). H9C2 cells were grown on cover slips and transfected with HA-3A αB-crystallin mutant using lipofectamine reagent according to the manufacturer's protocol. The cells, 48 h post-transfection, were given heat shock (43 °C for 1 h) and fixed immediately with 3.7% formaldehyde. These cells were stained with rhodamine-phalloidin for actin and with anti-HA mAb for HA-epitope.

2.2.4 Immunofluorescence Microscopy

H9C2 cells were grown on cover slips and given heat shock as described above. Cells were then washed twice with ice-cold cytoskeleton buffer (10 mM PIPES buffer, pH 7.0, containing 150 mM NaCl, 5 mM Glucose and 5 mM $MgCl_2$) and fixed with 3.7% formaldehyde. For recovery, heat-stressed cells were kept at 37 °C in a humidified CO_2 incubator. The fixed cells were permeabilized with 100% acetone at –20 °C for 8 min. After blocking with 2% BSA, cells were incubated with rabbit αB-crystallin polyclonal antibodies followed by incubation with Alexa-488 secondary antibody. For dual staining, the cells were incubated with rhodamine-phalloidin conjugate for 1 h to visualize F-actin after immunostaining for αB-crystallin. The cells were mounted in Vectashield medium containing DAPI. Confocal laser scanning microscopy was performed on a Carl Zeiss inverted microscope. Image analysis, including crossover subtractions and estimation of extent of co-localization, was done using LSM-FCS software (Version 5).

2.2.5 Immunoprecipitation

The immunoprecipitation experiments were performed essentially as described earlier (Wang *et al.,* 2005). H9C2 cells heat-stressed at 43 °C for 1 h, were lysed in 50 mM Tris-HCl buffer (pH 7.4) containing 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 0.2 mM Na_3VO_4, 0.2% NP40, 10 % glycerol for 15 min at 4 °C and centrifuged at 14000 rpm for 10 min. The supernatant containing soluble proteins was collected separately. The pellet was solubilized in 50 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 0.2 mM Na_3VO_4, 0.2% NP40, 1 % SDS, 0.5 % Triton X-100, 10 % glycerol and protease inhibitor cocktail (IP buffer). The supernatant and proteins solubilized from the pellet were incubated at 4 °C overnight with rabbit polyclonal αB-crystallin antibodies. Subsequently, protein-G magnetic beads were added to these samples and they were incubated further for 4 h. The magnetic bead complex was retrieved using a magnetic separator kit (Bangs Laboratories Inc. USA). The immunoprecipitated complex was washed thrice with ice cold IP buffer and suspended in Laemmli buffer. The samples were heated in a boiling water bath for 5 min, subjected to SDS-PAGE and processed for western blotting using appropriate antibodies.

2.2.6 F-actin Stability Test

H9C2 cells, grown on glass cover slips, were incubated either at 37 °C or subjected to heat stress at 43 °C for 1 h and further treated with cytochalasin B (3.0 μg/ml) for 15 min. After treatment, the cells were immediately fixed with 3.7% formaldehyde, immuno-stained using antibody specific for αB-crystallin and with rhodamine-phalloidin conjugate for F-actin, and analyzed by confocal microscopy. H9C2 cells not subjected to any of the above treatments served as controls. Image analysis, including crossover subtractions and estimation of extent of co-localization, was done using LSM-FCS software (version 5.0).

2.2.7 Treatment with Kinase Inhibitors

H9C2 cells grown on glass cover slips, were treated either with 20 μM SB202190 (a p38-MAP kinase inhibitor) or 50 μM PD098059 (a p44-MAP kinase inhibitor) for 18 h before and during the heat treatment. Heat-stressed and control cells were fixed with formaldehyde

for staining as described earlier. Concentrations of the vehicle, DMSO, used to dissolve the inhibitors, did not have any significant effect on the staining pattern.

2.2.8 SDS-PAGE and Western Blot Analysis

Heat-stressed and control cells were harvested and lysed in ice-cold lysis buffer, sonicated and centrifuged at 14000 rpm for 10 min at 4 °C. The supernatant represents the soluble fraction, and the pellet the insoluble protein fraction. Total cell lysates were prepared by solubulizing the cell pellet directly in Laemmli buffer. The total cell lysate, the soluble fraction and the insoluble fraction were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia). The membrane was blocked with 10% milk protein, sequentially incubated with appropriate primary antibodies and HRPO-conjugated secondary antibodies, and visualized by chemiluminescence (Amersham Biosciences, USA) as per the manufacturer's instructions.

2.2.9 Pinocytosis Analysis

H9C2 cells were grown on glass cover slips. Unstressed or heat-stressed (43 °C for 1 h) H9C2 cells were treated with cytochalasin B (3.0 μg/ml for 15 min) and further incubated with FITC-lysine dextran particles (10 mg/ml for 20 min). Unstressed or heat-stressed H9C2 cells were directly incubated with FITC-lysine dextran served as controls. The cells were then fixed with 3.7% formaldehyde and incubated with Hoechst-33258 for staining nuclei. Uptake of FITC-lysine dextran was visualized by confocal microscopy.

2.3 Results

2.3.1 Differential partitioning of αB-crystallin under heat stress

In order to investigate the partitioning of αB-crystallin between the soluble, cytosolic and the insoluble, cytoskeletal fractions upon heat shock; we have incubated H9C2 cells at 43 °C for 15, 30, 45, 60 or 75 min. The cells were lysed, the soluble and insoluble fractions separated and the levels of αB-crystallin determined by western blotting. We observed an increase in the level of αB-crystallin in the total cell lysate as the duration of heat shock increased (Figure 2.1A and D). When we analyzed the soluble and insoluble fractions of the cell lysates, αB-crystallin was found to be present in both the fractions.

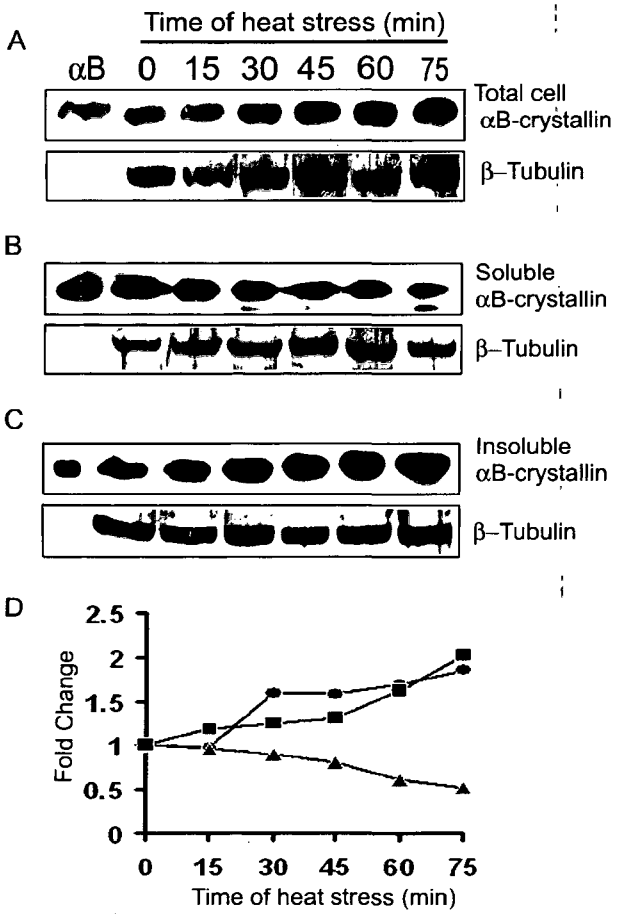


Figure 2.1. Western blot analysis to demonstrate the differential partitioning of αB-crystallin in H9C2 cells during heat stress. H9C2 cells, unstressed (0 min) or heat stressed at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min were lysed and fractionated into soluble and insoluble fractions as described in Materials and Methods. (A) Total cell lysate, (B) the soluble fraction, and (C) the insoluble fraction were immunoblotted for αB-crystallin. Lane αB shows the band corresponding to recombinant αB-crystallin that served as a positive control. β-Tubulins as loading controls are also shown. (D) Graphic representation of band intensities of αB-crystallin from total cell lysates (●), soluble (▲) and insoluble fractions (■) of unstressed and heat-stressed H9C2 cells, quantified by densitometry.

While we observed a decrease in the level of αB-crystallin in the soluble fraction as the duration of heat shock increased (Figure 2.1B and D), we found a gradual increase in its level in the insoluble fraction (Figure 2.1C and D).

2.3.2 αB-crystallin forms strands upon heat treatment

Since αB-crystallin increasingly partitioned to the insoluble fraction after heat stress, we analyzed its localization by confocal microscopy. Figure 2.2A shows that in unstressed H9C2 cells, αB-crystallin is predominantly found in the cytoplasm with almost no staining in the nucleus. After heat stress for 1 h (0 h recovery), αB-crystallin exhibits strand-like morphology in the cytoplasm and also migrates into the nucleus (Figure 2.2B). The formation of strands in the cytoplasm as well as the translocation of αB-crystallin into the nucleus seems to be reversible upon recovery. After 3 h of recovery, αB-crystallin is found to be uniformly distributed in the cytosol as well as in the nucleus (Figure 2.2C).

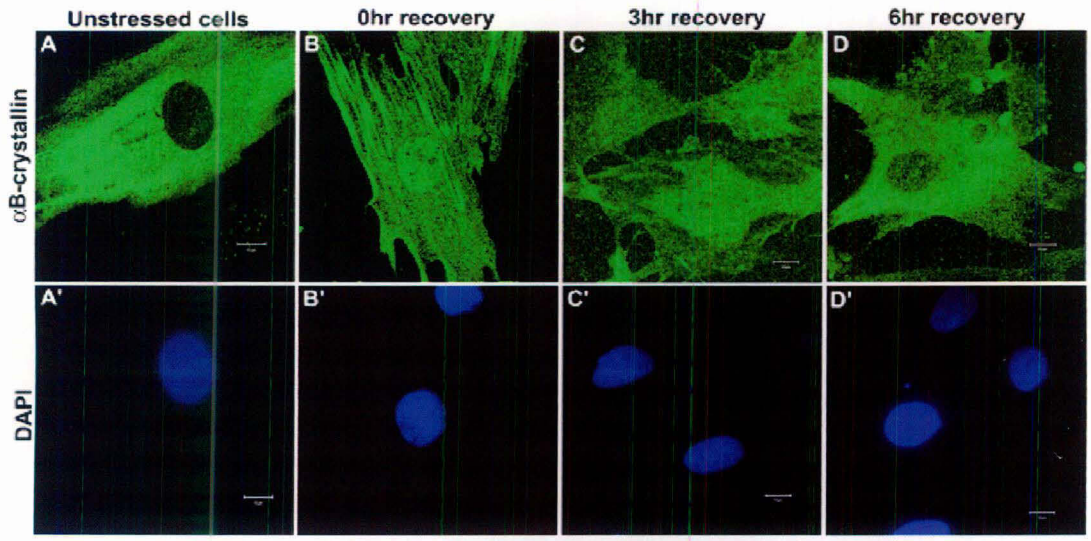


Figure 2.2. Immunolocalization of αB-crystallin upon heat stress. H9C2 cells, grown on coverslips were (A) unstressed or (B) subjected to heat stress at 43 °C for 60 min. The heat-stressed cells were allowed to recover at 37 °C for a period of (C) 3 h or (D) 6 h. (A)–(D) represent cells fixed in 3.7% (v/v) formaldehyde and immunostained using αB-crystallin-specific antibodies; (A')–(D') show the nuclei counterstained with DAPI. The scale bar represents 10 μm.

However, 6 h after recovery, most of the αB-crystallin translocates from the nucleus to the cytosol (Figure 2.2D). Our results showing that αB-crystallin partitions into the insoluble fraction (Figure 2.1) and forms fiber-like morphology upon heat stress (Figure 2.2B) suggest that αB-crystallin might associate with cytoskeletal proteins.

2.3.3 αB-crystallin associates with actin filaments *in vivo* upon heat stress

The actin microfilament network is an early target of cellular stress. It forms transient stress fibers, which disappear when the stress is released. It is likely that sHSPs such as αB-crystallin may contribute to the regulation of actin dynamics under normal and stressful conditions. We performed dual staining of H9C2 cells for αB-crystallin and actin after heat stress for various periods of time ranging from 15 min to 75 min and analyzed the extent of their co-localization by confocal microscopy. In unstressed cells, αB-crystallin is uniformly distributed in the cytosol and shows 23 % co-localization with actin fibers (Figure 2.3A). As the duration of heat stress increases, we observe increased fiber-like morphology of αB-crystallin as well as its increased co-localization with actin stress fibers (Figure 2.3C-F). After 75 min of heat stress, the extent of co-localization increased to 86 % (Figure 3F). At the earliest time point of heat stress studied (15 min), αB-crystallin also shows translocation into the nucleus (Figure 2.3B). Similar experiments were performed with primary cultures of neonatal rat ventricular myocytes (NRVMs). Under unstressed condition, αB-crystallin was found exclusively in the cytoplasm of NRVMs (Figure 2.4A). Interestingly, αB-crystallin exhibits strand-like appearance in the cytosol upon heat stress in these differentiated cells as well (Figure 2.4B). The cut mask panel shows the co-localized image of green and red pixels at the same point (Figure 2.4). Hence our data suggest that the nature of αB-crystallin is similar in both cardiomyoblasts (undifferentiated) as well as cardiomyocytes (differentiated). Arguably, it is important to note here that in differentiated cells, αB-crystallin does not migrate to the nucleus upon heat stress. Earlier studies from our laboratory have shown that αB-crystallin, which is mostly present in the cytoplasm, migrates to the nucleus upon stress, where it plays an important role in the stabilization of the nucleo-skeletal assembly (Adhikari *et al.,* 2004).

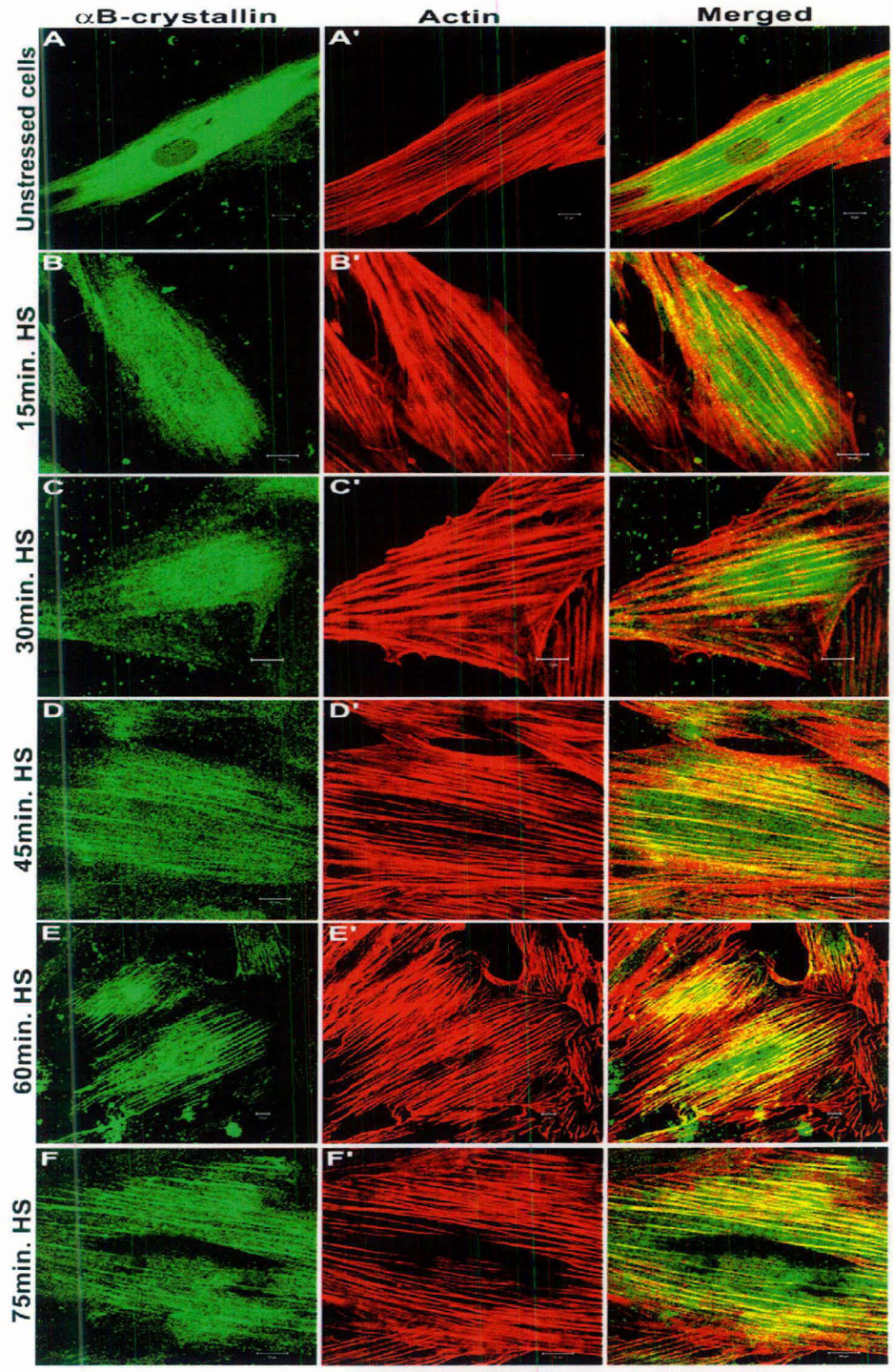


Figure 2.3. Co-localization of αB-crystallin with actin fibers in H9C2 cells. H9C2 cells, (A) unstressed or heat-stressed at 43 °C for (B) 15 min, (C) 30 min, (D) 45 min, (E) 60 min, or (F) 75 min were fixed with 3.7% (v/v) formaldehyde, permeabilized with chilled acetone and stained for αB-crystallin or for actin fibers. (A)–(F), represent αB-crystallin, shown in green and (A')–(F') actin fibers, shown in red. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. The scale bar represents 10 μm. The extent of co-localization was quantified using LSM-FCS software (version 5.0).

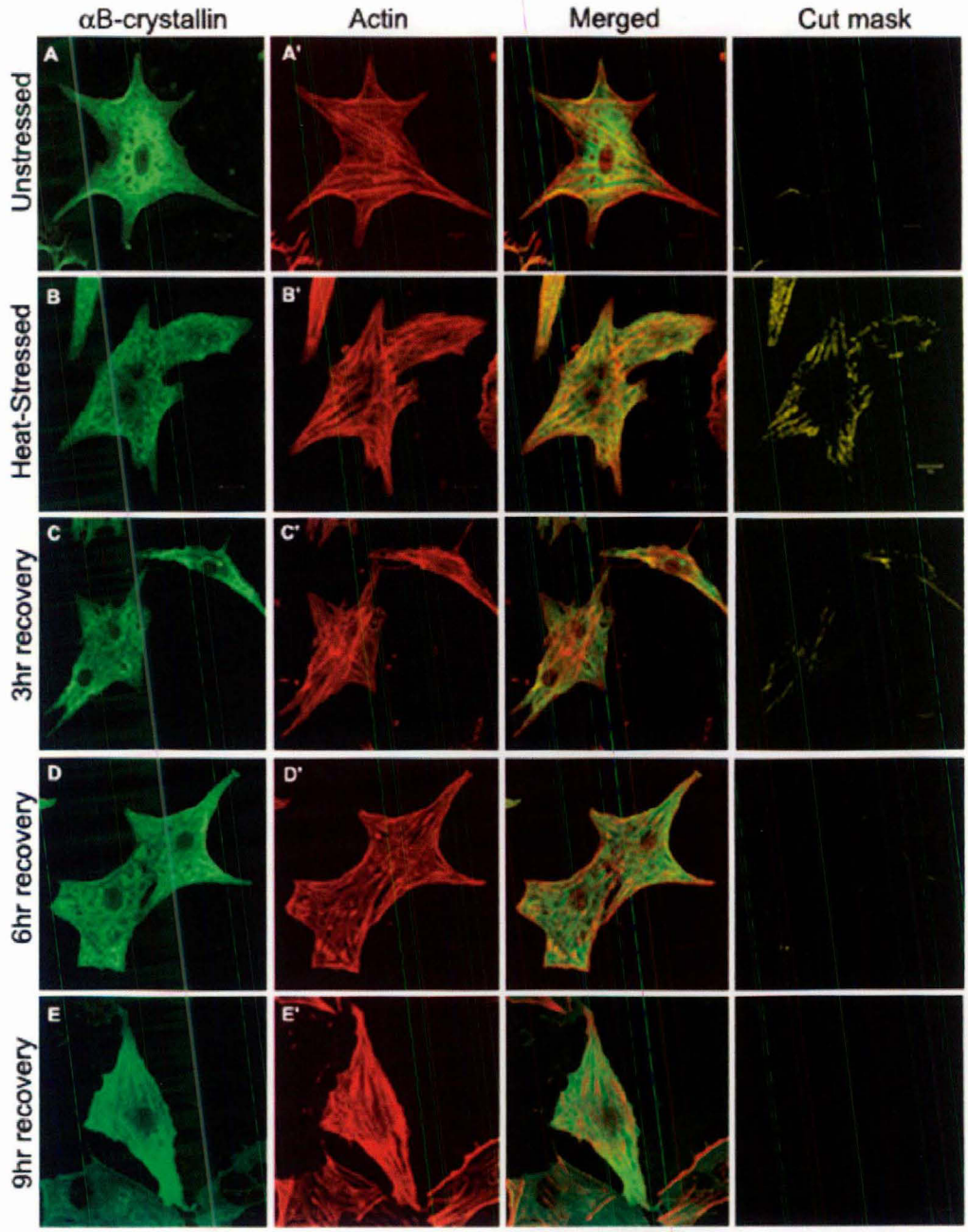


Figure 2.4. Co-localization of αB-crystallin with actin fibers in NRVMS. Neonatal rat ventricular cardiomyocytes, grown on coverslips were (A) unstressed or (B) subjected to heat stress at 43 °C for 60 min. The heat-stressed cells were allowed to recover at 37 °C for a period of (C) 3 h, (D) 6 h or (E) 9 h. (A)–(E) represent cells fixed in formaldehyde, permeabilzed with chilled acetone and immunostained using αB-crystallin-specific antibodies; (A')–(E'), actin fibers shown in red. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. Cut mask panel shows the colocalized region of green and red staining. The scale bar represents 10 μm. The extent of co-localization was quantified using LSM-FCS software (version 5.0).

Figures 2.2 and 2.3 show fiber-like morphology of αB-crystallin and its co-localization with actin filaments in the cytoplasm upon stress. In this chapter, we have investigated the importance of this association of αB-crystallin with actin filaments and its role in the organization of cyto-skeletal assembly.

The increased partitioning of αB-crystallin upon heat-stress to the insoluble fraction and its co-localization with actin filaments suggest that it may interact with actin under these conditions. We have subjected H9C2 cells to heat-stress at 43 °C for 1 h, lysed and performed immunoprecipitation using antibodies to αB-crystallin both in the soluble and insoluble fractions. Immunoblot analysis of this complex using antibody to actin (Figure 2.5, Upper Panel) shows detectable levels of actin in the soluble fraction of heat-stressed H9C2 cell lysates.

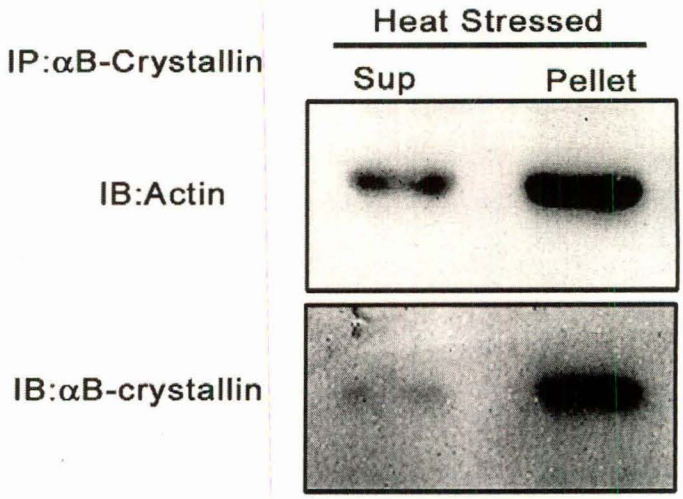


Figure 2.5. Immunoblot showing the interaction of αB-crystallin with actin. Lysates from heat-stressed H9C2 cells were fractionated into soluble and insoluble fractions. Proteins from the insoluble fraction solubilized in IP buffer (as described in Materials and Methods), and the soluble fraction were incubated with rabbit polyclonal αB-crystallin antibodies at 4 °C for 8 h. Subsequently, they were incubated with protein-G magnetic beads for 4 h. The proteins from the immunoprecipitated complexes were separated by SDS-PAGE (12% polyacrylamide gel) and transferred onto Nitrocellulose membrane. The membranes were immunoblotted with monoclonal actin antibodies (upper panel) or with monoclonal αB-crystallin antibodies (lower panel). Lane 1 shows bands from the soluble fraction and lane 2 shows the bands from the insoluble fractions.

Interestingly, the band intensity of actin in the immunoprecipitated complex from the insoluble fraction was significantly higher. Similarly, the αB-crystallin band was hardly detectable from the immunoprecipitated complex of the soluble fraction. As expected, the band intensity of αB-crystallin in the immunoprecipitated complex from the insoluble fraction

was significantly higher, suggesting the interaction of αB-crystallin with actin (Figure 2.5, Lower Panel).

2.3.4 Differential phosphorylation αB-crystallin upon heat stress

αB-crystallin is known to get phosphorylated at three serine residues (19, 45 and 59) *in vivo* and the extent of phosphorylation increases under conditions of stress (Smith *et al.*, 1992; Ito *et al.*, 1997; Hoover *et al.*, 2000). αB-crystallin has also been shown to regulate actin polymerization-depolymerization dynamics and stabilize them in a phosphorylation-dependent manner *in vitro* (Wang and Spector, 1996; Wieske *et al.*, 2001). In order to understand the role of the phosphorylation status of αB-crystallin in its interaction with actin filaments, we have analyzed the soluble and insoluble fractions after heat stress for different intervals of time. Using antibodies specific for Ser-59-phosphorylated αB-crystallin, we observed that the level of Ser-59-phosphorylated αB-crystallin increases 3.4 fold in the soluble fraction till 30 min of heat shock and gradually decreases thereafter (Figure 2.6A and C). On the other hand, the level of Ser-59-phosphorylated αB-crystallin in the insoluble fraction remains more or less constant until 30 min of heat stress and increases significantly from 45 min to 75 min (Figure 2.6B and C), the increase in the insoluble fraction being 2.8 fold at 75 min of heat shock.

Western blot analysis of Ser-45-phosphorylated αB-crystallin in the soluble fraction showed a gradual decrease and it reduced to 0.76 fold after 30 min of heat shock (Figure 2.7A and C), while its level in the insoluble fraction increased to 1.8 fold till about 60 min of heat stress after which there was a marginal decrease (Figure 2.7B and C). These results suggest that αB-crystallin gets differentially phosphorylated at different serine residues and may influence its association with actin stress fibers.

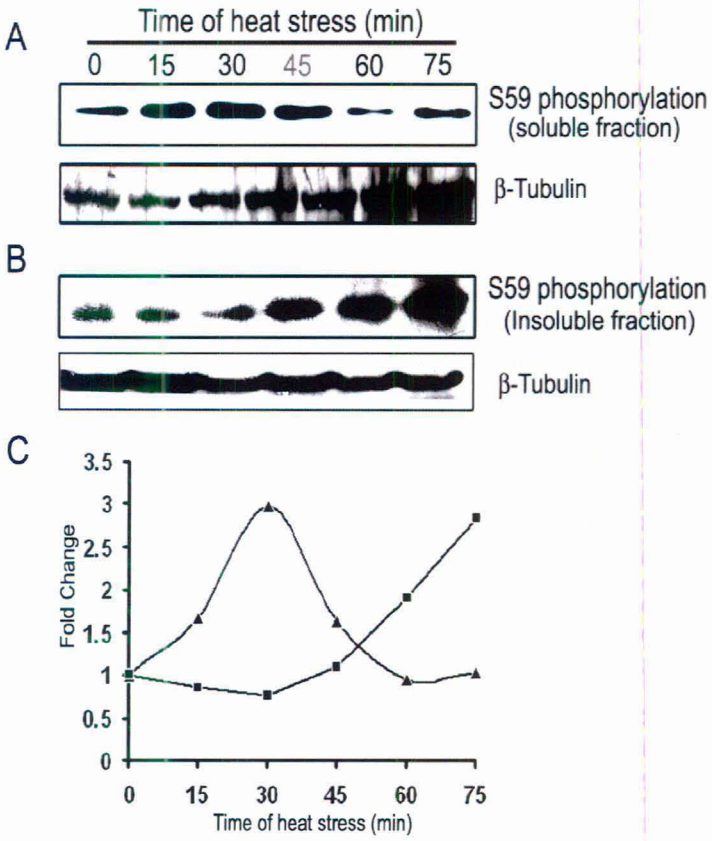
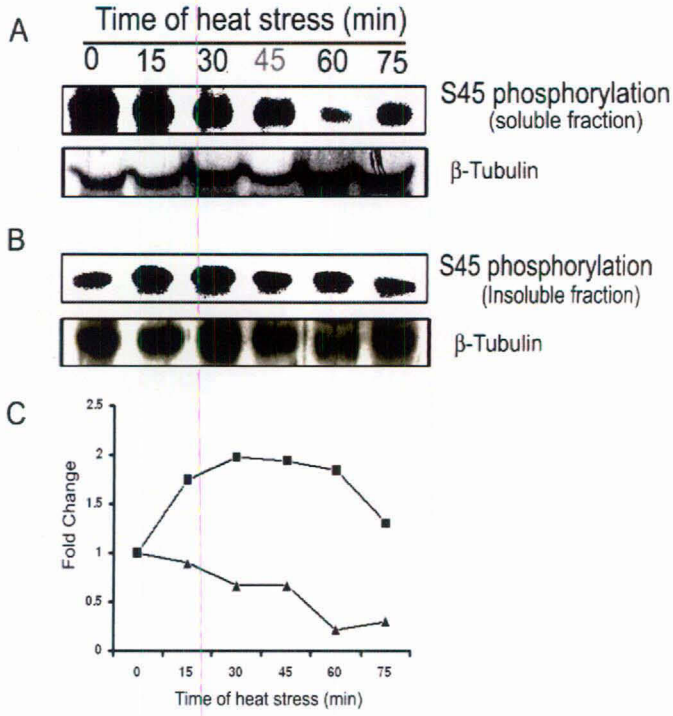


Figure 2.6. Western blots of Ser-59 phosphorylated αB-crystallin in the soluble and the insoluble fractions from lysates of unstressed and heat-stressed H9C2 cells. The soluble and insoluble fractions from lysates of unstressed H9C2 cells, or heat-stressed at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min were harvested as described earlier. Western blots show Ser-59-phosphorylated αB-crystallin in the soluble (A) and the insoluble (B) fractions. The β-tubulin bands as loading control are also shown. Figure 2.6C shows the graphic representation of band intensities as quantified by densitometry.

Figure 2.7. Western blots of Ser-45 phosphorylated αB-crystallin in the soluble and the insoluble fractions from lysates of unstressed and heat-stressed H9C2 cells. The soluble and insoluble fractions from lysates of unstressed H9C2 cells, or heat-stressed at 43 °C for 15 min, 30 min, 45 min, 60 min or 75 min were isolated as described earlier. Western blots show Ser-45-phosphorylated αB-crystallin in (A) the soluble and (B) the insoluble fractions. The β-tubulin bands as loading control are also shown. Figure 2.7C shows the graphic representation of band intensities as quantified by densitometry.



2.3.5 Association of αB-crystallin with actin filaments during heat stress depends upon its phosphorylation

In order to study the role of phosphorylation of αB-crystallin in its association with actin stress fibers, we have transiently transfected H9C2 cells with a HA-tagged non-phosphorylatable mutant of αB-crystallin (the three serine residues are substituted by alanine). Our data shows that the distribution of the 3A-mutant is similar to that seen for wild type αB-crystallin in unstressed cells (Figure 2.8A). However, unlike wild type αB-crystallin, the 3A-mutant fails to co-localize with actin stress fibers even after heat stress for 60 min (Figure 2.8B). Thus, phosphorylation of αB-crystallin appears to be important for its association with actin stress fibers.

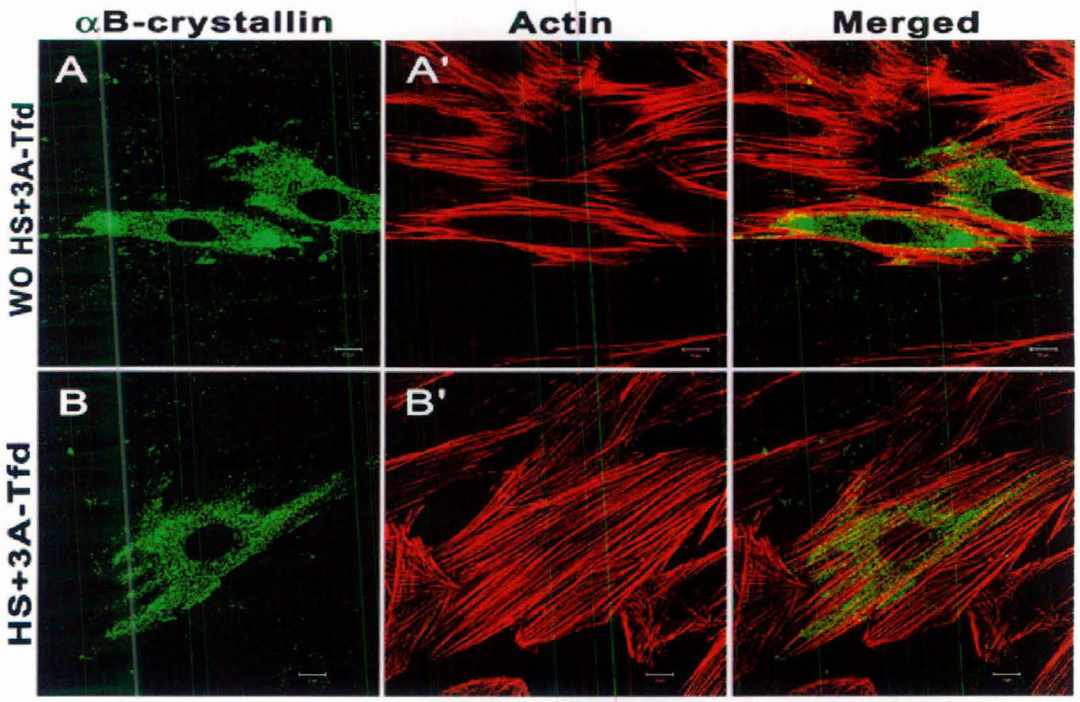


Figure 2.8. Phosphorylation status of αB-crystallin affects its association with actin fibers. H9C2 cells grown on glass coverslips were transfected with a non-phosphorylatable HA-3A-mutant of αB-crystallin. The transfected cells, either unstressed or heat-stressed at 43 °C for 60 min, were fixed and stained with anti-HA antibodies for the mutant αB-crystallin and with rhodamine-phalloidin for actin fibers. (A) and (A') Staining for the HA-epitope and actin fibers in unstressed cells. (B) and (B'), the staining pattern for the HA-epitope and actin fibers in cells subjected to heat-stress. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. The scale bar represents 10 μm.

We have also used specific inhibitors of the p44 (PD98059) and p38 (SB202190) MAP kinases that have earlier been shown to be involved in the site-specific phosphorylation of Ser-45 and Ser-59 residues respectively (Ito *et al.,* 1997). Our results show that inhibition of phosphorylation at Ser-59 (Figure 2.9A) as well as Ser-45 (Figure 2.9B) of αB-crystallin almost completely inhibits its association with actin stress fibers. Thus our study suggests that the association of αB-crystallin with actin filaments is phosphorylation-dependent, and that phosphorylation of both Ser-45 and Ser-59 is essential for this association.

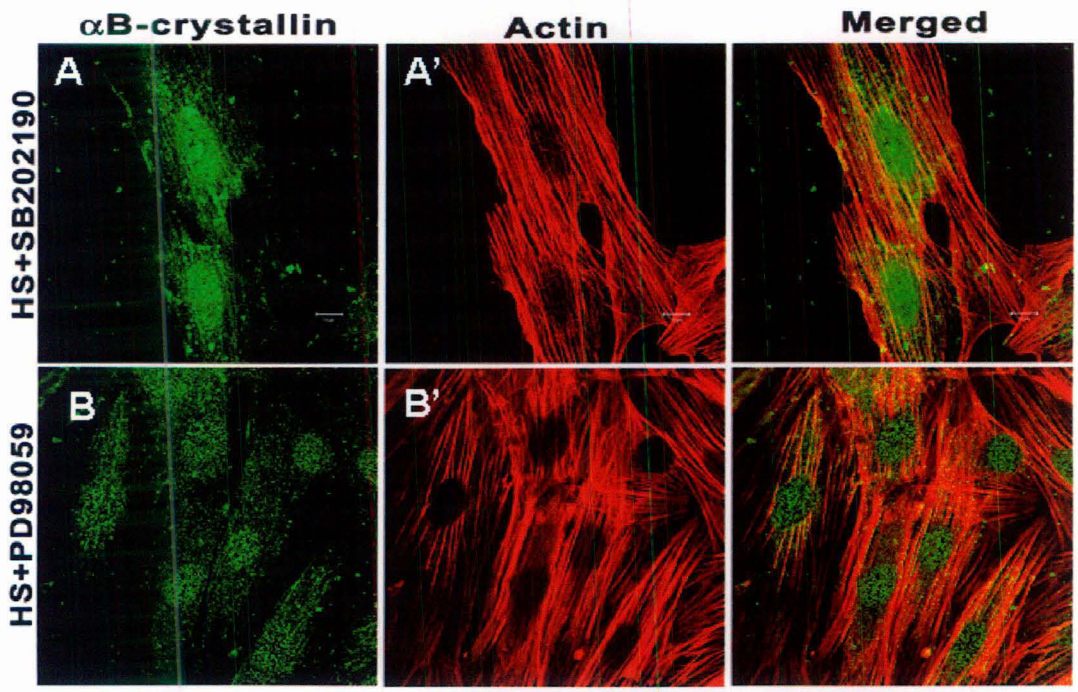


Figure 2.9. Effect of MAPK inhibitors on the association of αB-crystallin with actin fibers. H9C2 cells were treated with SB202190 (p38 MAPK inhibitor) or PD098059 (p44 MAPK inhibitor) for 18 h and later subjected to heat stress at 43 °C for 60 min and fixed with 3.7% (v/v) formaldehyde permeabilized and stained with rabbit polyclonal antibodies for αB-crystallin and with rhodamine-phalloidin for actin fibers. (A) and (B) The staining pattern for αB-crystallin or (A') and (B') for actin after treatment of cells with SB202190 and or PD098059, respectively. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. The scale bar represents 10 μm.

Importantly, pretreatment of cells with MAPK inhibitors, and subsequently subjected to heat stress followed by cytochalasin B treatment led to disorganization of actin microfilaments (Figure 2.10). This data further corroborates our finding that the non-phosphorylated αB-crystallin does not associate with filamentous actin and phosphorylation

is essential for the association of αB-crystallin with F-actin (Figure 2.10). As shown in figure 2.10A' and B', inhibition of phosphorylation of αB-crystallin using specific inhibitors leads to cytochalasin B-induced disorganization of F-actin even in heat-stressed cells. Further, the fiber-like appearance of αB-crystallin was not observed in the heat-stressed cells suggesting that the fiber morphology of αB-crystallin was due to the decoration of αB-crystallin along the actin fibers. Our data also indicates that the association of phosphorylated αB-crystallin with F-actin is protective in nature and prevents the depolymerization of F-actin during heat stress.

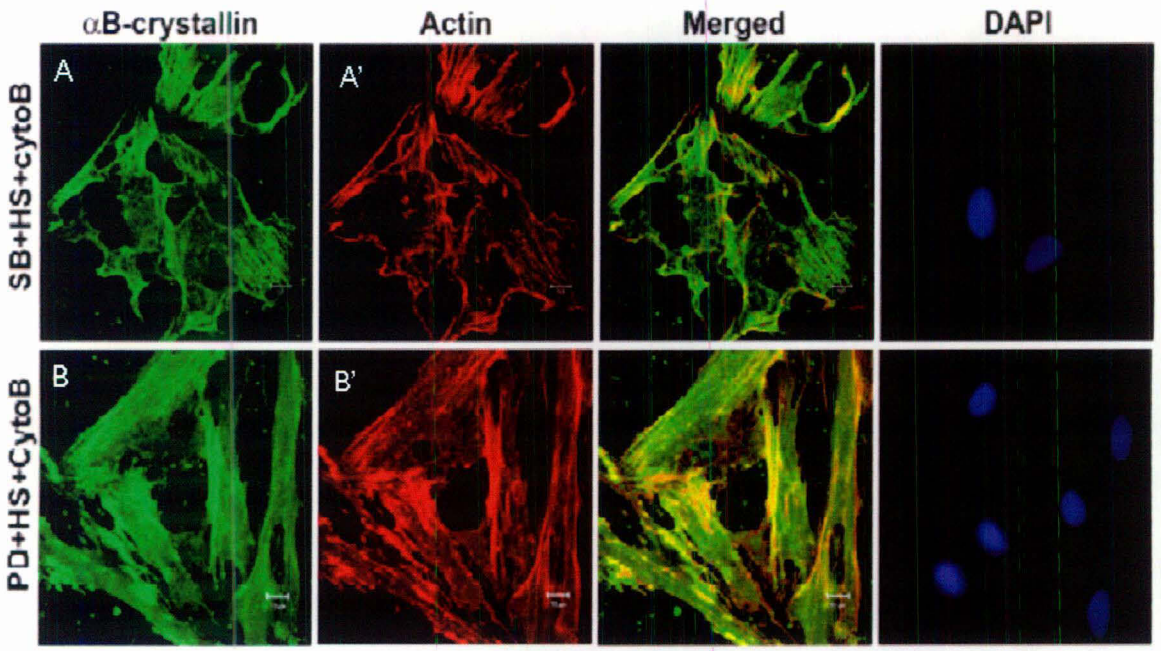


Figure 2.10. Effect of MAPK inhibitors on the association of αB-crystallin with actin fibers. H9C2 cells were treated with SB202190 (p38 MAPK inhibitor) or PD098059 (p44 MAPK inhibitor) for 18 h and later subjected to heat stress at 43 °C for 60 min and followed by treatment with cytochalasin B (3 μg/ml). Cells were fixed with 3.7% (v/v) formaldehyde permeabilized and stained with rabbit polyclonal antibodies for αB-crystallin and with rhodamine-phalloidin for actin fibers. (A) and (B) The staining pattern for αB-crystallin or (A') and (B') for actin after treatment of cells with SB202190 and PD098059, respectively followed cytochalasin B treatment in heat-stressed cells. Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. The scale bar represents 10 μm

2.3.6 Functional significance of αB-crystallin association with actin filaments

We have earlier shown that αB-crystallin stabilizes actin microfilaments by associating with them and hence prevent their disorganization. To investigate the functional

significance of this association, we treated unstressed or heat-stressed H9C2 cells with cytochalasin B (3.0 μg/ml), an inhibitor of actin polymerization, and studied its effect on the association of αB-crystallin with actin stress fibers. In unstressed cells, αB-crystallin has cytoplasmic localization (Figure 2.2A and 2.11A). Figure 2.11B shows that actin stress fibers are significantly reduced in H9C2 cells not subjected to heat stress but treated with cytochalasin B. Under these conditions, the degree of co-localization of αB-crystallin with actin is minimal.

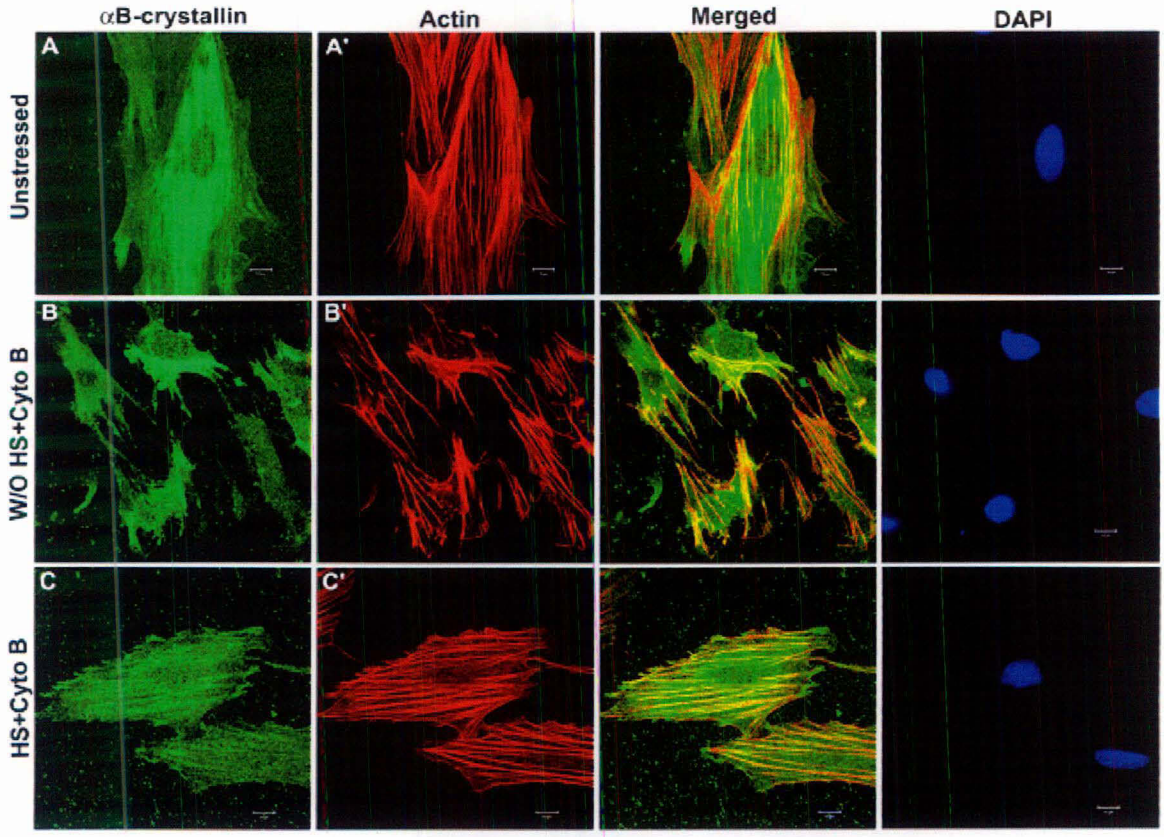


Figure 2.11. Effect of cytochalasin B, on the association of αB-crystallin with actin fibers. H9C2 cells, unstressed or heat stressed at 43 °C for 60 min, were subsequently incubated with 3 μg/ml of cytochalasin B for 15 min, fixed with 3.7% (v/v) formaldehyde, permeabilized and stained for αB-crystallin (A)–(C) or for actin fibers (A')–(C'). Merged panels show the overlays of confocal images of dual-stained cells in a single optical section of 0.3 μm. The scale bar represents 10 μm.

On the other hand, when the cells are subjected to heat stress and then treated with cytochalasin B, cells retain stress-fiber morphology and also show significant association

with αB-crystallin (Figure 2.11C). Thus, our results suggest that αB-crystallin prevents heat-induced disorganization and helps in the maintenance of filamentous architecture of actin.

2.3.7 Pinocytosis Analysis

The results obtained so far demonstrate that association of αB-crystallin with actin stress fibers protects the cytoskeletal network and prevents heat-induced damage to the cells. Cellular processes like pinocytosis and cell motility involve extensive polymerization and depolymerization of actin microfilaments (Theriot and Mitchison, 1991; Nakase *et al.*, 2004). We, therefore, investigated the possible role of αB-crystallin in regulating physiological activities such as pinocytosis during heat stress as reflected by the uptake of FITC-dextran particles (Klein and Satre, 1986). Cytochalasin B treatment is known to dissociate actin polymers and hence interfere with several physiological activities including pinocytosis (Wagner *et al.*, 1997).

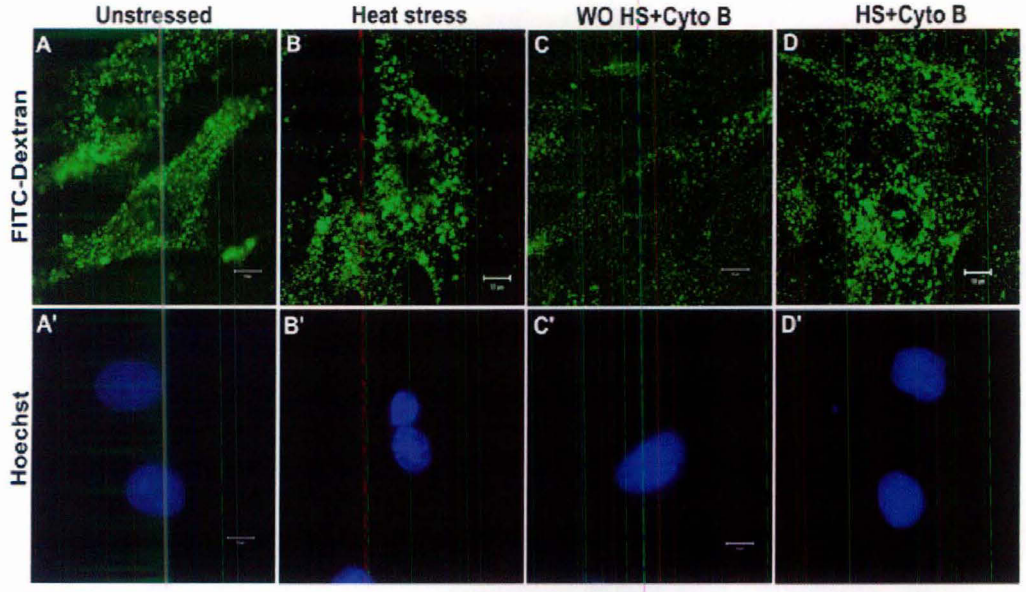


Figure 2.12. Role of αB-crystallin in pinocytosis. Pinocytosis in H9C2 cells was studied by the uptake of FITC-lysine dextran particles and visualized by confocal laser microscopy. H9C2 cells, (A) unstressed and (C) treated with 3.0 μg/ml of cytochalasin B for 15 min or (B) heat-stressed at 43 °C for 60 min and (D treated with cytochalasin B were subsequently incubated with 10 mg/ml of FITC-lysine dextran for 20 min. The cells were fixed with 3.7% (v/v) formaldehyde and analyzed by confocal microscopy. (A')–(D') The nuclei stained with Hoechst 33258. The scale bar represents 10 μm.

Unstressed H9C2 cells, when incubated with FITC-lysine dextran, a significant number of dextran particles were observed in the cytoplasm of the cell, indicating the uptake of the FITC-lysine dextran particles (Figure 2.12A). The uptake of the FITC-dextran particles in heat-stressed was increased to ~1.57 fold in comparison to normal unstressed cells (Figure 2.12B). Interestingly, unstressed cells when treated with cytochalasin B, the uptake of FITC-lysine dextran was decreased to approximately 0.54 fold (Figure 2.12C and Table 2.1) compared to that by normal, untreated cells (Figure 12A). Importantly, the uptake of FITC-particles in cells subjected to heat stress and subsequently treated with cytochalasin B was 1.4 fold (Table 2.1), showing no significant decrease (Figure 2.12D) in comparison to heat-stressed cells.

Table 2.1: Uptake of FITC-dextran particle by H9C2 cells

Treatments	Total fluorescence Intensity	Fold difference from unstressed cells
Unstressed cells	392.75	1.00
Heat stressed cells	618.7	1.57
Unstressed Cytochalasin B-treated cells	214.8	0.54
Heat Stressed Cytochalasin B-treated cells	551.25	1.4

Thus, the enhanced association of αB-crystallin with actin fibers in heat-stressed H9C2 cells reduces the inhibitory effects of cytochalasin B on pinocytosis. Our results, therefore, suggest that in addition to protecting the organization of the cytoskeletal network, the association of αB-crystallin with actin fibers also helps in maintaining the functional integrity of cells subjected to stress.

2.4 Discussion

αB-crystallin, a member of small heat shock protein family, has been implicated in prevention of aggregation of non-native proteins and also in several diverse cellular functions such as cell division, differentiation and apoptosis (Wang and Spector, 1995; Raman *et al.*, 1995; Ito *et al.*, 2001a; Bai *et al.*, 2004; Kamradt *et al.*, 2005). Studies have shown that αB-crystallin interacts with proteins of cytoskeleton and modulates their dynamics *in vitro* and *in vivo* (Djabali *et al.*, 1997; Perng *et al.*, 1999). The association of αB-crystallin with cytoskeletal proteins alters under conditions of stress and helps in maintenance and regulation of their dynamics (Nicholl and Quinlan, 1994). Actin, a cytoskeletal protein, is highly susceptible to stressors such as heat stress and acidic environment (Glass et al., 1985; Ganote and Vander Heide, 1987). Bennardini *et al.* (1992) demonstrated that αB-crystallin could interact with actin and desmin filaments *in vitro* and their binding affinity increases after heat treatment. However, such interaction of αB-crystallin with actin filaments *in vivo* and the consequences of this interaction in stress protection are not clearly understood. Here, we report the *in vivo* interaction of αB-crystallin with actin filaments, temporal changes during heat stress and its protective role. We find that this interaction of αB-crystallin prevents heat-induced disorganization of actin filaments. Thus, our results suggest an important role for αB-crystallin in the maintenance of cellular functions such as pinocytosis.

Our present data together lends further support to the view that αB-crystallin plays a significant role in protection of the cytoskeletal organization. Figure 2.1 shows the differential partitioning of αB-crystallin into soluble and insoluble fraction in a time-dependent manner; under conditions of stress αB-crystallin preferentially partitions into insoluble fraction. Dual staining confocal image analysis shows that αB-crystallin appears as cytoplasmic strands and aligns with actin stress fibers (Figure 2.2 and Figure 2.3). The formation of strands in the cytoplasm upon heat stress and the co-localization of αB-crystallin with actin filaments clearly demonstrate its association with cytoskletal elements *in vivo*. Further, immunoprecipitation experiments have shown that αB-crystallin interacts with actin filaments (Figure 2.5). Earlier studies have shown that the interaction of αB-crystallin with actin filaments *in vitro* is phosphorylation-dependent (Wang and Spector, 1996).

Phosphorylation of αB-crystallin seems to play a major role in its localization, interaction with target proteins and its cellular functions (Morrison *et al.*, 2003; den Engelsman *et al.*, 2004; den Engelsman *et al.*, 2005). Western blot analysis using Ser-59 and Ser-45 phospho-specific antibodies show that heat stress leads to phosphorylation of these residues. Interestingly, we observed stress-induced temporal changes in the partitioning of phosphorylated αB-crystallin from soluble to insoluble fractions, the partitioning being dependent on the phosphorylation of serine-59 and 45 residues (Figure 2.6 and Figure 2.7). This data suggests that upon stress, αB-crystallin gets phosphorylated at Ser-59 residues and subsequently it binds to actin filaments thereby depleting its concentration in the soluble fraction. On the other hand, concentration of αB-crystallin phosphorylated at Ser-45 decreases gradually in the soluble fraction while in the insoluble fraction it increases till 45 min and decreases thereafter. The reason for the observed decrease in Ser-45 phosphorylated αB-crystallin from the insoluble pool is not clear. Phosphorylation of serine residues is necessary for αB-crystallin binding to actin. In contrast, phosphorylation of HSP27 leads to its dissociation from actin (Miron *et al.*, 1991). Further studies are necessary to investigate whether dephosphorylation of αB-crystallin might have any role in its dissociation from the actin. Phosphorylation leads to extensive redistribution of αB-crystallin upon heat stress in a time-dependent manner. It appears that phosphorylation at Ser-45 or Ser-59 is essential for its interaction with actin fibers, since inhibition of phosphorylation at either of these sites using specific inhibitors, abrogates this interaction. Inhibition of αB-crystallin phosphorylation by MAP kinase inhibitors (SB202190 and/or PD98059) may affect other proteins as well and could indirectly result in the failure of αB-crystallin to localize with stress fibers. The fact that non-phosphorylatable 3A-αB-crystallin fails to associate with actin filaments even in the absence of MAP kinase inhibitors, suggests that the association of αB-crystallin with actin is phosphorylation-dependent (Figure 2.8 and Figure 2.9). It is possible that phosphorylation makes αB-crystallin competent to associate with actin filaments. Interestingly, we also observed that both phosphorylated αB-crystallin and the 3A mutant exhibit similar oligomeric size (lower than αB-crystallin) in a glycerol density gradient centrifugation using H9C2 cell lysates (data not shown). This result implies that change in size *per se* is not sufficient for the association of αB-crystallin with actin filament. Rather, it appears that phosphorylation and hence its effects, if any, on the local conformation may be important for such interaction. Our studies also show that αB-crystallin protects the filamentous architecture of actin fibers from

cytochalasin B-induced depolymerization (Figure 2.11). This protection seems to be phosphorylation-dependent as upon treatment of cells with p38/p44 MAP kinase inhibitors, the cellular architecture is lost and very few actin filaments were seen (Figure 2.10). Launay *et al.* (2006) have reported that treatment of cells with agents that disturb cytoskeleton can lead to activation of p38 MAPK and phosphorylation of Ser-59 residue of αB-crystallin, which in turn protects the cytoskeleton. Similar results have been shown for HSP27, where the regulation of the F-actin dynamics is p38 MAP kinase-depedent (Guay *et al.*, 1997); whether these two sHSP function in a coordinated or independent manner is not yet clear. It has also been shown that αB-crystallin interacts with microtubule associated proteins and protects the microtubules from nocodazole- or calcium-induced disassembly (Fujita *et al.*, 2004). We speculate that αB-crystallin might form a coat around F-actin and prevent cytochalasin B-induced disorganization of the actin stress fiber architecture. Stress fiber formation and membrane ruffling are associated with increased pinocytosis, an essential cellular function (Bar-Sagi and Feramisco, 1986; Chow *et al.*, 1998). We have investigated the effect of heat stress and cytochalasin B in the pinocytotic process. We find that unstressed, cytochalasin B-treated cells show dramatically reduced pinocytosis compared to heat preconditioned, cytochalasin B-treated cells as reflected by the uptake FITC-dextran particle (Figure 2.12). Heat stress effects on pinocytosis have been shown for other cells such as B-cells and dendritic cells. In these cells increased pinocytosis leads to increased antigen presentation (Cristau *et al.*, 1994). Pinocytosis plays a significant role in nutrient and drug uptake in several types of cells (Jirmanova *et al.*, 1977; Van Deurs and Nilausen, 1982). Our studies suggest that αB-crystallin maintains the cellular architecture by stabilizing actin stress fibers and helps in the preservation of functional integrity of H9C2 cells subjected to heat. Altered physico-chemical properties and dynamics of actin filaments can lead to acute myocardial infarction and heart failure (Nishida *et al.*, 1987). Hence association of αB-crystallin with actin stress fibers may perform a crucial role in maintaining structural and functional integrity of cardiac tissue and help in prevention of myocardial cell death.

CHAPTER 3

Role of αB-crystallin in Muscle Differentiation

3.1 Introduction

αB-Crystallin, a member of the small heat shock protein (sHSP) family, originally thought to be a lenticular protein, is expressed in several non-lenticular tissues such as brain, heart and kidney (Bhat and Nagineni, 1989; Klemenz *et al.,* 1991). αB-Crystallin acts as a molecular chaperone and prevents the stress-induced aggregation of target proteins (Horwitz, 1992; Rao *et al.*, 1993; Raman and Rao, 1994; Wang and Spector, 1995). Studies from our laboratory have shown that αB-crystallin undergoes structural alteration and display enhanced chaperone activity at higher temperature (Raman and Rao, 1994; Raman *et al.,* 1995). We have shown that in heat-stressed C2C12 myoblasts, αB-crystallin migrates to the nucleus where it colocalizes with lamins and SC-35 (Adhikari *et al.,* 2004). Our recent finding using H9C2, rat cardiomyoblasts, show that αB-crystallin associates with filamentous actin during heat-stress (Singh *et al.,* 2007). The association of αB-crystallin with cytoskeletal and nucleoskeletal proteins suggests its cytoprotective function under conditions of stress. In addition to its chaperone functions, αB-crystallin has been shown to play an important role in many cellular events such as proliferation, differentiation and apoptosis (Kato *et al.,* 1998; Ito *et al.*, 2001a; Bai *et al.*, 2004a; Kamradt *et al.,* 2005).

Muscle differentiation is a dynamic process, which is characterized by the proliferation of myoblasts, followed by the cell-cycle arrest and terminal differentiation into multi-nucleated mature muscle fiber (Clegg *et al.,* 1987; Lassar *et al.,* 1994). In the normal proliferating condition, myoblasts express significant amount of αB-crystallin as well as other sHSPs such as HSP27, HSPB2/B3, and HSPB6 (p20) (Boelens *et al.,* 1998; Sugiyama *et al.,* 2000). These sHSPs are shown to form different complexes during muscle differentiation and are involved in myogenic differentiation and maintenance (Sugiyama *et al.,* 2000). During muscle differentiation, the expression of sHSPs such as αB-crystallin, MKBP/HSPB2 and HSPB3 are induced and their expression has been shown to be under the control of MyoD (Sugiyama *et al.,* 2000). Interestingly the increase in expression of other sHSPs during the muscle differentiation process is not as high as that of αB-crystallin; while the level of HSP27 gets up-regulated by 3-fold; that of αB-crystallin is enhanced by 10-fold (Ito *et al.,* 2001a). Importantly, knockout studies show that mice lacking αB-crystallin have lower muscle mass (Brady *et al.,* 2001). A point mutation in αB-crystallin, R120G is known to be associated with desmin related myopathy (DRM), where it forms aggregates with the desmin

intermediate filaments (Vicart *et al.,* 1998; Sanbe *et al.,* 2004). All these findings clearly demonstrate the critical role of αB-crystallin in muscle differentiation and its maintenance.

The regulation of skeletal muscle differentiation is essential for the normal development and any alterations in this process may lead to pathological conditions such as muscular dystrophies and inflammatory myopathies (Lundberg and Dastmalchi, 2002; Ferrari and Mavilio, 2002). Skeletal muscle differentiation is regulated by sequential and coordinated expression of a family of muscle-specific basic helix-loop-helix (bHLH) transcription factors called as Muscle Regulatory Factors (MRFs), which include MyoD, Myf5, myogenin and MRF4 (Weintraub, 1993). When ectopically expressed, these MRFs have unique property of converting non-muscle cells into muscle lineage (Weintraub *et al.,* 1989; Choi *et al.,* 1990). It is shown that mice lacking myogenin fail to terminally differentiate, whereas MRF4 is required for the proper maintenance of the differentiated myotubes (Hasty *et al.,* 1993; Kassar-Duchossoy *et al.,* 2004). MyoD and Myf5 are expressed in the proliferating undifferentiated myoblasts and are shown to be involved in the specification of myogenic lineage (Braun and Arnold, 1996). MyoD is also required for the healthy self-renewing proliferation of adult skeletal muscle satellite cells (Megeney *et al.,* 1996). Mice deficient in MyoD have severe impairment in their ability to regenerate muscle tissue after tissue injury (Yablonka-Reuveni *et al.,* 1999; Cooper *et al.,* 1999). MyoD, upon activation, induces cell cycle arrest by enhancing the p21 promoter activity (Halevy *et al.,* 1995). Terminal cell cycle arrest is coupled to muscle differentiation and is required for the activation of muscle-specific gene expression (Maione and Amati, 1997; Walsh and Perlman, 1997). Exit from the cell-cycle, therefore, is critical for the muscle differentiation program. Another important parameter that governs myogenesis is the ability of myoblasts to survive during differentiating conditions. Kamradt *et al.,* (2002), have shown that αB-crystallin protects myoblasts by preventing the activation of caspase-3 during differentiation. Various studies have reported that αB-crystallin binds to p24 (a pro-active caspase-3) upon TNF-α treatment (Kamradt *et al.,* 2001). Recently, our laboratory has demonstrated that over expression of αB-crystallin in C2C12 cells protects them from TNF-α induced cytotoxicity by enhancing the activity of NF-κB, an ubiquitous transcription factor (unpublished data). The activation of NF-κB by αB-crystallin is intriguing as NF-κB is known to control cell proliferation and differentiation through the transcriptional regulation of cyclin D1 (Guttridge *et al.,* 1999). The cyclic expression of cyclin D1 regulates cell division by complexing with cdk4/6, which further phosphorylates and inactivates pRB at the G1/S

transition (Kato *et al.,* 1993a; Resnitzky *et al.,* 1994). It is found that the over-expression of cyclin D1 results in an inhibition of MyoD-dependent transcription and decrease in p21 expression (Rao *et al.,* 1994; Skapek *et al.,* 1995). Thus, it appears that the decision to differentiate relies on the cross-talk between MyoD and cell-cycle signaling pathways. Whether αB-crystallin, by modulating NF-κB activity, alters cyclin D1 profile and thereby influences the muscle differentiation program needs to be investigated. Recently Di Carlo *et al.,* (2004) have shown that hypoxia leads to inhibition of muscle differentiation through accelerated degradation of MyoD protein. It is important to note that during hypoxic condition, αB-crystallin levels goes up by many folds and it also exhibits protective function (Tumminia and Russell, 1994; Nefti *et al.,* 2005). It is not clear if the increased level of αB-crystallin modulates the differentiation process during hypoxic condition? In addition, it is recently shown that over-expression of αB-crystallin using adeno-viruses leads to defects in myogenesis (Ikeda *et al.,* 2006). All these reports are suggestive of important role for αB-crystallin in the myogenic differentiation. The molecular mechanisms of its involvement in muscle differentiation, however, are not understood.

This chapter describes our investigations on the role of αB-crystallin in muscle differentiation using C2C12, mouse myoblasts as a model system. We demonstrate that the regulation of αB-crystallin is critical for proper myogenesis. We find that αB-crystallin delays the muscle differentiation process to a significant extent. Our study suggests that the delay in the differentiation seems to be the combined effect of alteration in cell-cycle exit and modulation of myogenic regulator, MyoD.

3.2 Materials and Methods

3.2.1 Antibodies and Reagents

DMEM, Fetal Calf Serum (FCS), DMSO, MTT and mouse monoclonal anti-Flag antibodies were purchased from (Sigma Chemical Company, USA). Cycloheximide, MG132, rabbit polyclonal anti-Ubiquitin antibodies were obtained from Calbiochem, EMD Biosciences, Germany. Propidium Iodide (PI), Alexa-488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes, Invitrogen Corp. Oregon, USA. Anti-GAPDH mouse monoclonal antibodies were purchased from Chemicon International Inc., USA. Rabbit polyclonal antibodies for αB-crystallin, phosphoserine-59-αB-crystallin and phosphoserine-45-αB-crystallin and mouse monoclonal anti-cyclin D1 antibodies were obtained from Stressgen Biotechnologies, Victoria, Canada. Mouse monoclonal antibodies for MyoD were purchased from Dako Cytomation, USA; rabbit polyclonal anti-myogenin antibodies were from Santa Cruz, USA. Mouse monoclonal antibodies for p21 and phospho-p38 MAPK were from BD Transductions, Pharmingen, USA. Protease inhibitor cocktail (PIC) was procured from Roche Applied Sciences, USA; Lipofectamine-2000 was obtained from Invitrogen, USA. HRPO-conjugated anti-rabbit, anti-mouse secondary antibodies and Enhanced Chemiluminescence (ECL) Western blot detection kit were purchased from Amersham Biosciences, USA. Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, USA.

3.2.2 Plasmids and Construction of FLAG-tagged cDNAs

cDNA of the full-length αB-crystallin was PCR-amplified using the following primers: **Forward primer**- 5'- GGCCGAATTCATGGACATCGCCATCCACCAC-3'and **Reverse primer**- 5'-GCCCTCGAGCTATTTCTTGGGGGCTGCGG-3'. The PCR products were digested with EcoR I and Xho I restriction enzyme and ligated into a modified pCDNA3 vector, in frame with the FLAG epitope, which was inserted upstream of the multiple cloning site. The final positive clones were confirmed by automated DNA sequencing.

3.2.3 Cell culture and Differentiation

C2C12, mouse skeletal myoblast cell line, was maintained at sub-confluent densities (60-70%) in DMEM supplemented with 20% fetal calf serum (FCS), 100 units/ml of penicillin and100 μg/ml of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. To induce differentiation, cells at 89-90% confluence were shifted to DMEM supplemented with 2% horse serum, (differentiating medium (DM)). At different time-points of differentiation, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), containing 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 0.2 mM Na_3VO_4, 0.2% (v/v) NP-40 and Protease Inhibitor Cocktail (PIC). For immunoflourescence studies, cells were grown on coverslips and allowed to differentiate for different lengths of time, as indicated. Cells were fixed with 3.7% (v/v) formaldehyde after each time-point and processed for confocal analysis. The differentiation index (DI) and fusion index (FI) was calculated as described in the text.

3.2.4 Stable Transfections

For stable transfections, C2C12 cells were grown in six well plate and transfected either with 1μg pCDNA3 vector alone or with 1μg of pCDNA3-N FLAG-tagged-αB-crystallin plasmids using Lipofectamine 2000 reagent (Invitrogen Corp., USA). At 48h post-transfection, the cells were sub-cultured in medium containing geneticin (Roche Applied Sciences, USA) and grown for a period of one month. Subsequently, stably transfected clones of αB-crystallin were selected in the presence of 500 μg/ml of G418 (Geneticin) (Invitrogen Corp., USA). Single cell clones were isolated and the ones expressing not less than two fold higher levels of αB-crystallin (as determined by western blot analysis using anti-Flag antibodies) were expanded and used for our study. C2C12 cells that stably over-expressed αB-crystallin were referred to as CRYAB-C2C12.

3.2.5 Cell Survival Assay

C2C12 cells and cells over-expressing αB-crystallin (CRYAB-C2C12) were grown on cover slips till 80-90% confluency. Subsequently, cells were shifted to differentiating medium (DM) and allowed to undergo differentiation for different time periods. Cells were washed twice with PBS and incubated with MTT (50 μg/ml) for 4 hrs. DMSO was added to dissolve the formazan crystals and OD was measured at 570 nm. The graph was plotted as optical density (570 nm) versus time of differentiation.

3.2.6 FACS Analysis

C2C12 cells and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. At each time point, the cells were fixed in 80% methanol, stained with propidium iodide, and cell cycle analysis was done using Fluorescence Activated Cell Sorter, (Facs Caliber-Becton and Dickinson USA). The values represent average of three independent experiments and are expressed as percentage.

3.2.7 Immunofluorescence Microscopy

C2C12 cells and cells over-expressing αB-crystallin (CRYAB-C2C12) were grown on cover slips till 80-90% confluence. Subsequently, cells were shifted to differentiating medium (DM) and allowed to undergo differentiation for different time periods. At each time point, cells were washed twice with ice-cold PBS and fixed with 3.7% formaldehyde. The fixed cells were permeabilized with 0.05% Triton X-100 for 8 min. After blocking with 2% BSA, cells were incubated with antibodies specific for myogenin and αB-crystallin, followed by incubation with Alexa-488- and Cy3-tagged secondary antibody respectively. The cells were mounted in Vectashield medium containing DAPI. Confocal laser scanning microscopy was performed on a Carl Zeiss inverted microscopy. Image analysis was done using LSM 510 meta software (Version 5).

3.2.8 SDS-PAGE and Western Blot Analysis

C2C12 cells and CRYAB-C2C12 cells were induced to undergo differentiation in DM and harvested at respective time points. Cells were lysed in ice-cold lysis buffer, sonicated and centrifuged at 14000 rpm for 10 min at 4 °C. Equal amount of protein was loaded on 12% SDS-polyacrylamide gels. After electrophoresis, proteins were transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia). The membrane was blocked with 10% (w/v) milk protein and incubated sequentially with appropriate primary antibodies and HRPO-conjugated secondary antibodies and was visualized using ECL kit (Amersham Biosciences, USA) according to the manufacturer's instructions. The band intensities were quantified by densitometry using GeneTools software (Syngene). The ratio of band intensities of the respective blots and that of the corresponding

loading control, expressed in arbitrary units was used for comparison. The values represent an average of a minimum of three independent experiments.

3.2.9 Comparison of Protein Synthesis Rate

C2C12 cells and CRYAB-C2C12 cells were allowed to differentiate for 12 h and 24 h respectively and subsequently incubated with MG132 (10 μM) to inhibit the 26S proteasomal degradation pathway. Following incubation for 0, 1, 2, and 3h, cells were harvested and lysed in ice-cold cell lysis buffer and cell lysates were collected as described above. Equal amount of proteins were subjected to SDS-PAGE and western blot analysis was performed as described previously followed by its densitometric analysis. The pixels for each band were measured and normalized so that the number of pixels at t = 0 was 1. The pixels of each band were plotted *versus* time. Protein synthesis rates were compared based on the initial slope from plots of data from 0 to 3 h.

3.2.10 Determination of Apparent Protein Half-life

As described above, C2C12 cells and CRYAB-C2C12 cells were induced to differentiate for 24 h, following which, cells were incubated with cycloheximide (CHX, 50 μg/ml, Sigma) to inhibit further protein synthesis. The 26S proteasomal inhibitor MG132 (N-benzyloxycarbonyl-Leu-Leu-leucinal, 10 μM, Calbiochem) was added along with CHX when necessary. Following incubation for 0, 0.5, 1, 2, and 3 h, cells were lysed in lysis buffer, and equal amounts of total cell protein were subjected to SDS-polyacrylamide gel electrophoresis. Western blotting was performed as described above using antibodies specific for MyoD. The pixels fo6r each band were measured and normalized so that the number of pixels at $\boldsymbol{t = 0}$ was 100%. Protein degradation rate is expressed as apparent half-life ($\boldsymbol{t_{1/2}}$), the time for degradation of 50% of the protein from its initial value. The $\log_{10}$ of the percentage of pixels was plotted *versus* time, and the $\boldsymbol{t_{1/2}}$ was calculated from the $\log_{10}$ of 50%.

3.2.11 Immunoprecipitation

C2C12 and CRYAB-C2C12 cells were differentiated using differentiating medium (DM) for various time points. The cells lysates were lysed in 50 mM Tris-HCl buffer (pH 7.4)

containing 50 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 0.2 mM Na_3VO_4, 0.2% NP40, 10 % glycerol (IP buffer) at 4 °C followed by mild sonication with two pulses of 10 seconds duration. Supernatants were collected by centrifugation at 14 000 rpm for 10 min. The total cell extract was incubated at 4 °C overnight with rabbit polyclonal anti-ubiquitin antibodies. Subsequently, protein-G magnetic beads were added to these samples and they were incubated further for 4 h at 4 °C. The magnetic bead complex was retrieved using a magnetic separator kit (Bangs Laboratories Inc. USA). The immunoprecipitated complex was washed three times with ice-cold IP buffer and suspended in Laemmli buffer. The samples were heated in a boiling water-bath for 5 min, subjected to SDS-PAGE and processed for Western blotting using appropriate antibodies and then detected using the ECL system (Amersham Pharmacia, USA).

3.3 Results

3.3.1 αB-crystallin alters myogenic differentiation

To delineate the possible role of αB-crystallin during myogenic differentiation, C2C12 myoblasts and cells stably over-expressing αB-crystallin (CRYAB-C2C12) were allowed to differentiate for different time periods and analyzed for their ability to undergo myogenic differentiation. The phase-contrast micrographs show that C2C12 myoblasts fused into myotubes and extensive myotube formation was observed at 72 h of differentiation.

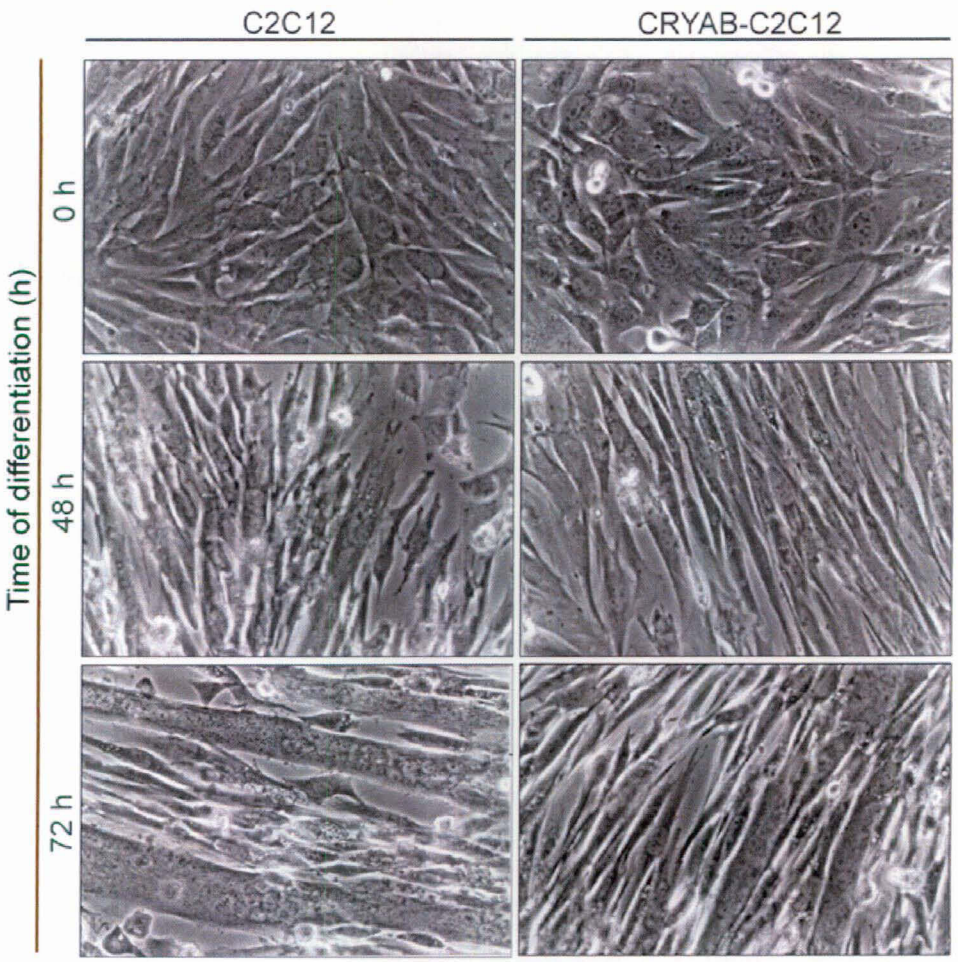


Figure 3.1: αB-crystallin alters myogenic differentiation. C2C12 cells and cells over-expressing αB-crystallin (CRYAB-C2C12) were grown to 90-100% confluency in growth medium (GM). Subsequently, cells were allowed to differentiate in DMEM containing 2% horse serum (DM). Phase contrast images were taken at different time-points at a magnification of 200X using inverted microscope, Nikon, Japan. The images obtained were further processed using adobe photoshop version 6.0.

Surprisingly, at 72 h of differentiation, the myotube formation in CRYAB-C2C12 cells was poor compared to that in C2C12 cells (Figure 3.1).

To gain further insight into the delay in myogenic differentiation, we calculated the differentiation (DI) and fusion indices (FI) as described by Sabourin *et al.*, (1999) using the following formulae;

$$DI = \frac{\text{No. of myogenin positive cells}}{\text{No. of nuclei}} \qquad FI = \frac{\text{No. of fused cells}}{\text{No. of nuclei}}$$

The differentiation and fusion indices were calculated as per the above formulae and representated in percentage as shown in figure 3.2. We find that the differentiation index (DI) is significantly less in CRYAB-C2C12 cells; at 72 h of differentiation it is ~ 50% compared to ~75% in C2C12 cells (Figure 3.2A). Similarly the fusion index (FI) is also significantly less; it is ~25% in CRYAB-C2C12 cells compared to ~50% in myoblasts at the same time point (Figure 3.2B).

Our results showing impairment in the myotube formation (Figure 3.1) and reduction in the differentiation as well as fusion index (Figure 3.2) suggest that the differentiation process is severely affected in CRYAB-C2C12 cells. The impairment in the fusion process could also arise from the enhanced death of myoblasts during the differentiation process. In order to test this possibility, we have carried out cell viability assay using MTT. As can be seen from the figure 3.3 both C2C12 and CRYAB-C2C12 cells display similar survival profile, thereby excluding the possibility of cell death influencing the process of myogenesis in CRYAB-C2C12 cells.

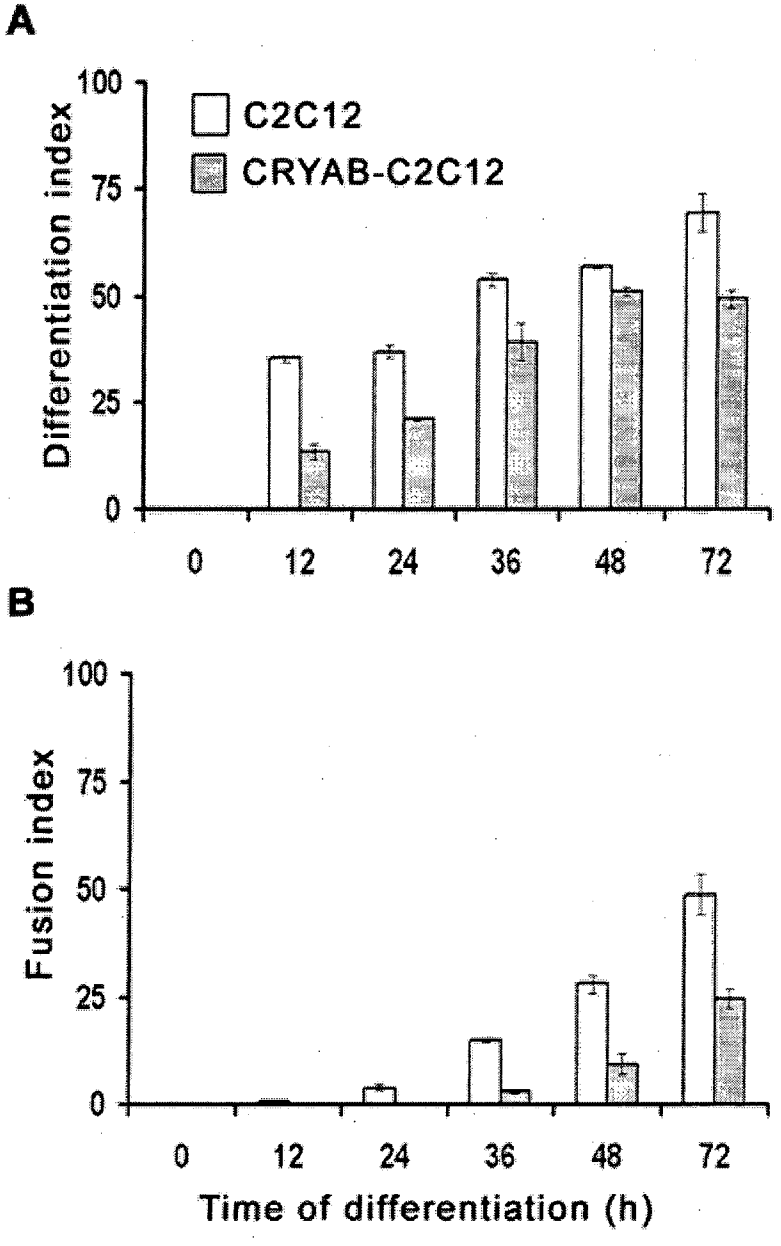


Figure 3.2: αB-crystallin affects differentiation index and fusion index during differentiation. C2C12 and CRYAB-C2C12 cells growing on coverslips were induced to differentiate by shifting the cells in DMEM medium containing 2% horse serum. Cells were fixed and immunostained using specific antibody to myogenin at various time-points. **Panel A** shows the Differentiation index (DI) of C2C12 and CRYAB-C2C12 cells. The DI was calculated based on the total number of myogenin positive cells versus total number of nuclei. **Panel B** shows the Fusion index (FI) of C2C12 and CRYAB-C2C12 cells. The FI was calculated based on the total number of fused cells (≥ 2 nuclei) versus total number of nuclei.

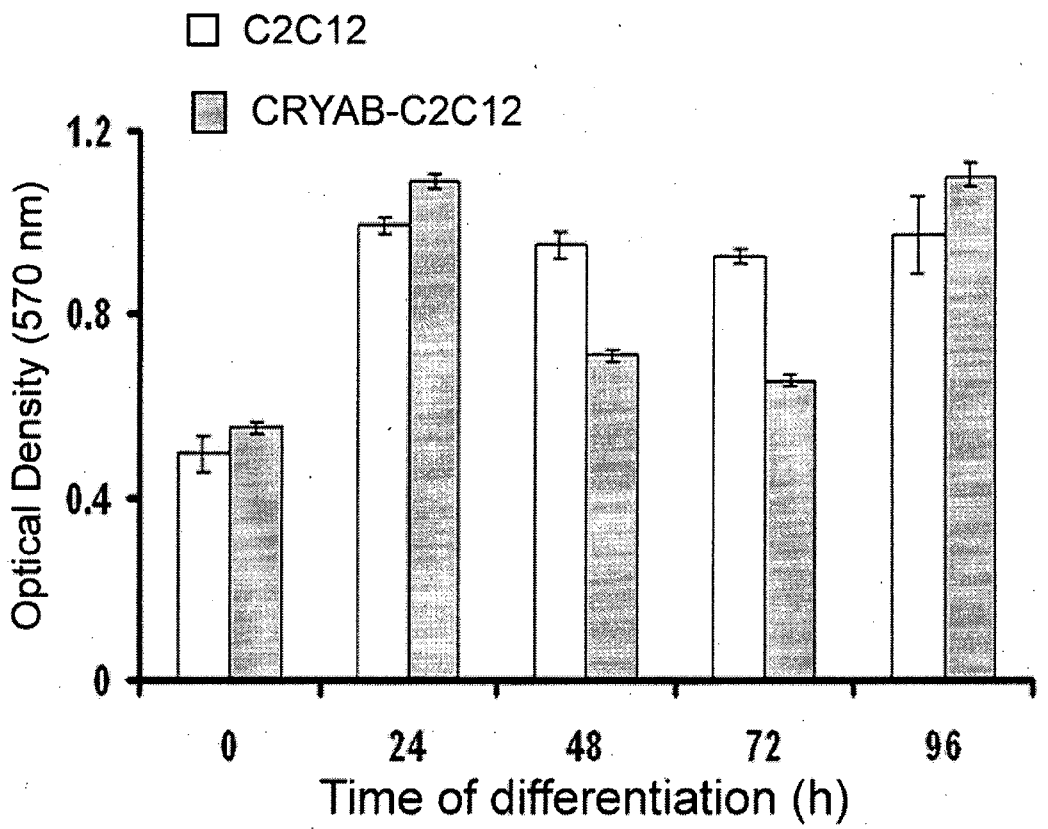


Figure 3.3: MTT assay for cell survival in C2C12 and CRYAB-C2C12 cells. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM containing 2% horse serum for different period of time. At each time point, cells were incubated with MTT (50 µg/ml) and the formazan crystals were dissolved by adding 100% DMSO. Optical density was measured at 570 nm and normalized with 0 h time point. Bar diagram was plotted as cell survival (%) versus time of differentiation (h).

3.3.2 αB-crystallin maintains myoblasts in the proliferative phase

Exit from cell-cycle is critical for myogenic transcription and completion of the differentiation program (Walsh and Perlman, 1997). To decipher the mechanism of delay in myogenesis, we compared the cell-cycle profiles, of C2C12 and CRYAB-C2C12 myoblasts. FACS analysis of myoblasts induced to differentiate for different time points, fixed in 80% ethanol and stained with propidium iodide (PI), showed that ~ 38 % of the C2C12 cells were in G1-phase of cell cycle compared to 45% in case of CRYAB-C2C12 cells initially upon induction of differentiation (0 h time point). Twelve hours after induction of differentiation, we find that C2C12 cells undergo G0/G1 arrest (~ 60%). We observed a concomitant and significant decrease in the population of G2/M pool from ~21% before induction to 1.45% at 12 h of differentiation.

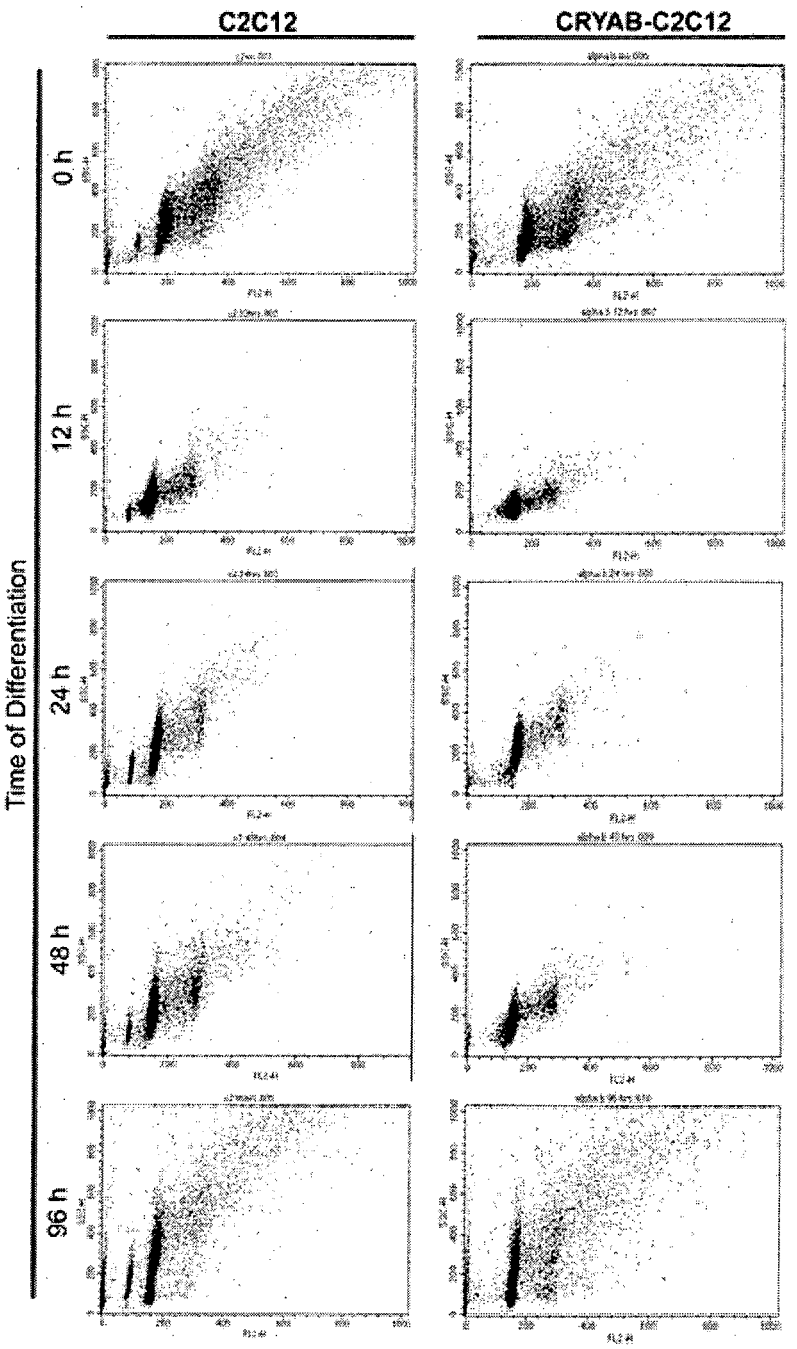


Figure 3.4: FACS profile of C2C12 and CRYAB-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. Differentiated cells were fixed in 80% methanol and further stained with Propidium Iodide (PI) and analyzed by fluorescence activated cell sorter (FACS). The scatter plot describes the cell cycle profile of C2C12 and CRYAB-C2C12 cells during differentiation. The open circle represents the G0 population in C2C12 cells, whereas the densely dotted area shows the G1-arrested cell population.

Interestingly, CRYAB-C2C12 cells show significantly less number of G0/G1 arrested cell population (~ 23%) at 12 h time point, while the G2/M population was comparable to that of C2C12 cells. Importantly, the G0-arrested cells (subG1) also include a pool of apoptotic cells, which occurs during the differentiation process. The relatively less percentage of subG1 population of CRYAB-C2C12 cells compared to that of C2C12 cells at 12 h time point of differentiation is suggestive of the protective function of αB-crystallin. At 24 h and 48 h of differentiation, the subG1 populations of C2C12 cells were higher than that of CRYAB-C2C12 cells. It is important to note that at 96 h of differentiation, greater percentage of CRYAB-C2C12 cells (~ 7.96%) were in G2/M phase compared to those in C2C12 cells (~ 4.96%). This indicates that αB-crystallin maintains the cells in the proliferative stage and delays cell-cycle arrest (Figure 3.4 and Table 3.1).

		Sub-G1	G1	G2/M
0 h	C2C12	5.77±0.29	38.3±1.83	20.48±0.49
	CRYAB-C2C12	3.26±0.09	45.25±1.49	23.45±1.23
12 h	C2C12	59.78±0.59	14.27±0.71	1.55 ±0.27
	CRYAB-C2C12	23.25±1.15	56.23±1.19	1.67 ±0.19
24 h	C2C12	11.08±0.34	66.48±2.38	3.44 ±0.19
	CRYAB-C2C12	3.51 ±0.39	86.72±2.21	4.44 ±0.16
48 h	C2C12	45.97±1.61	15.88±0.83	3.03 ±0.21
	CRYAB-C2C12	9.48 ±0.94	69.91±0.79	3.37 ±0.29
96 h	C2C12	12.13±0.68	36.52±0.94	4.96 ±0.12
	CRYAB-C2C12	3.23 ±0.14	44.08±0.78	7.96 ±1.06

Table 3.1: FACS profile of C2C12 and CRYAB-C2C12 cells during differentiation.

To further dissect out the molecular details, we set out to investigate the cell-cycle regulators, cyclin D1 and p21. Cyclin D1 and p21 function with opposite effects- cyclin D1 induces G1/S transition whereas p21 is involved in the cell-cycle arrest by inhibiting the CDKs (Nurse, 1994; Sherr, 1994). To find out whether αB-crystallin affects withdrawal from the cell-cycle, we monitored the expression of cyclin D1 and p21 at different time points after induction of differentiation. Our western blot analysis shows a significant expression of cyclin D1 before the induction of differentiation, in C2C12 cells, following which its level decreases sharply to 0.12 fold at later stages of differentiation (Figure 3.5A and 3.5C).

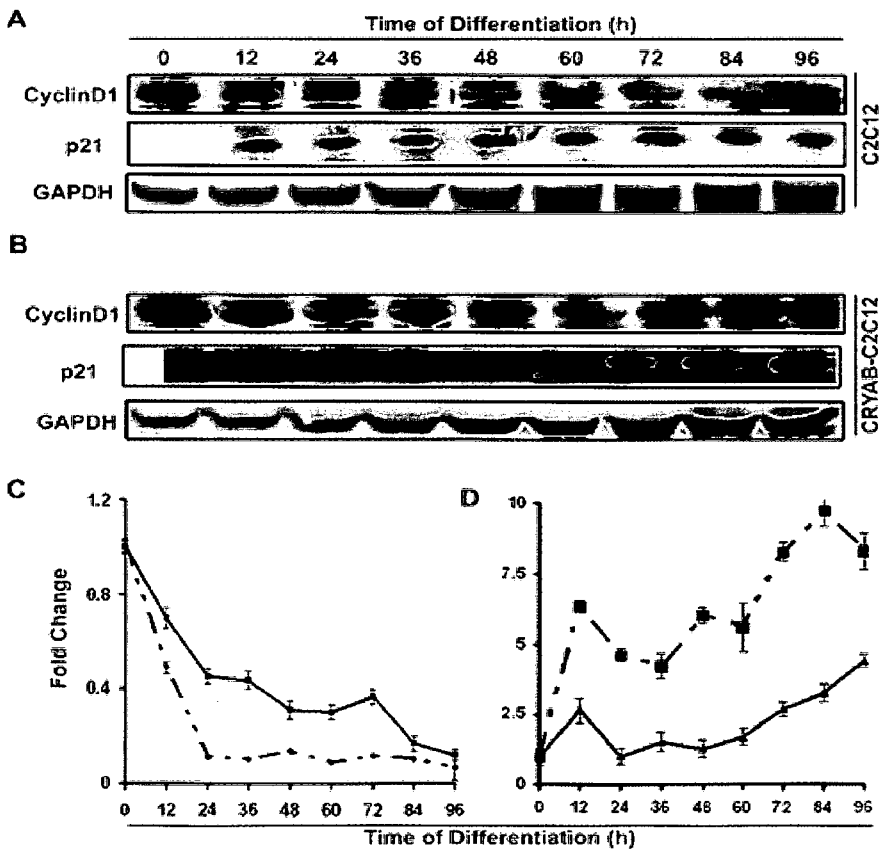


Figure 3.5. Western blots showing cyclin D1 and p21 profile during C2C12 and CRYAB-C2C12 cells differentiation. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** and **panel B** shows western blots for cyclin D1 and p21 profile from C2C12 and cells overexpressing αB-crystallin (CRYAB-C2C12) cell lysates. Western blot for GAPDH, used as loading control is also shown. **Panel C** shows the graphic representation of cyclin D1 profile from C2C12 (▲) and CRYAB-C2C12 (■) cell lysates respectively and **panel D** shows the graphic representation of band intensities of p21 in C2C12 (■) and CRYAB-C2C12 (▲)

As expected, there was a gradual increase in p21 expression upon induction of differentiation which increased to 8.3 fold at 96 h of differentiation in C2C12 cells (Figure 3.5A and 3.5D). In contrast to C2C12 myoblasts, the level of cyclin D1 in CRYAB-C2C12 cells was high initially upon differentiation (0 h time point). Interestingly, a significant level of cyclin D1 was sustained till 36 h of differentiation after which it decreased gradually (Figure 3.5B and 3.5C). Moreover, there was a delayed onset of p21 expression in CRYAB-C2C12 cells, and was detectable only after 60 h of differentiation. The expression level of p21 increased gradually to 4.1 fold at 96 h of differentiation in CRYAB-C2C12 cells (Figure 3.5B and 3.5D).These data suggest that the sustained level of cyclin D1 and delay in the p21 expression together alter the cell-cycle profile in CRYAB-C2C12 cells (Figure 3.4 and Figure 3.5).

3.3.3 αB-crystallin negatively regulates the expression of myogenic markers

The results reported in the previous sections prompted us to investigate whether delay in the cell-cycle arrest, could affect the expression of muscle regulatory genes. To analyze the delay in the differentiation process, we checked the expression of differentiation markers desmin and myogenin. Our western blot analysis shows that the expression of myogenin gets induced upon differentiation and was detectable as early as 12 h of differentiation in C2C12 cells (Figure 3.6A and 3.6B). However, the expression of myogenin was severely affected in CRYAB-C2C12 cells; significant increase was detected only after 60 h of differentiation (Figure 3.6A and 3.6B).

Further, immunostaining for myogenin show that myogenin-positive cells were rarely seen in the early stages of differentiation (12 h) in CRYAB-C2C12; where as a significant population of C2C12 cells were expressing myogenin at similar time points (Figure 3.6C). Interestingly, the staining for myogenin was comparable in both cell types at later stages of differentiation (72 h). This suggests that αB-crystallin delays but does not inhibit the expression of myogenic regulators during the differentiation process.

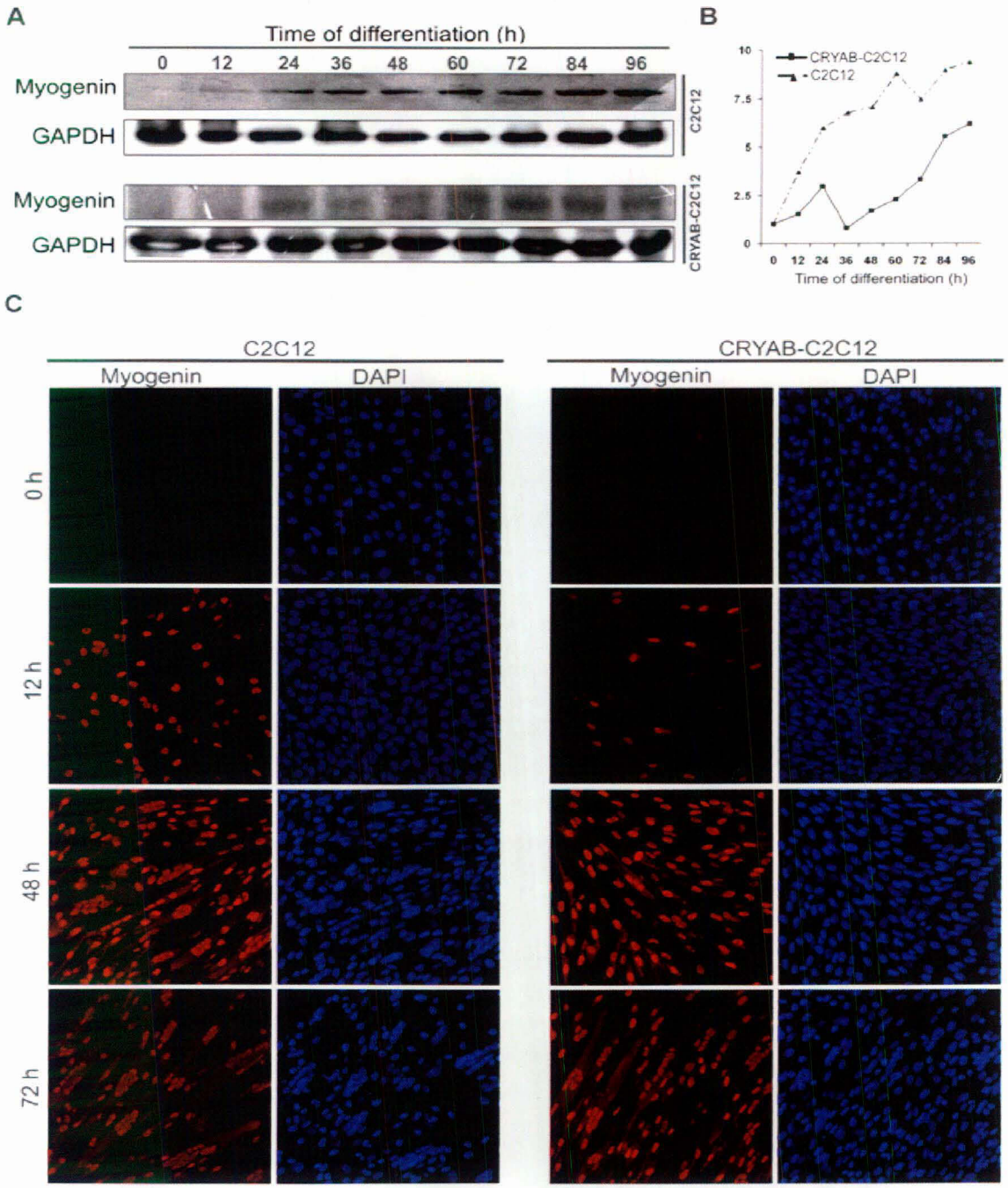


Figure 3.6. Profile of the myogenic marker, myogenin in C2C12 and CRYAB-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** shows the western blots for myogenin expression profile from lysates of C2C12 cells and cells overexpressing αB-crystallin (CRYAB-C2C12). Western for GAPDH, used as loading control is also shown. **Panel B** describes the graphic representation of band intensities of myogenin from cell lysates of C2C12 (▲) and CRYAB-C2C12 (■) cells. **Panel C** shows the immunostaining for myogenin in C2C12 and CRYAB-C2C12 cells at different time periods of differentiation.

Similarly, the expression of desmin was severely affected and it appeared only after 72 h of differentiation in CRYAB-C2C12 myoblasts compared to 48 h of differentiation in C2C12 cells (data not shown). Together, our data shows that αB-crystallin affects cell-cycle exit and induces delay in the expression of regulatory proteins involved in the differentiation process.

3.3.4 αB-crystallin alters MyoD level during muscle differentiation

During myogenesis, the expression of both myogenin and p21 is known to be regulated by MyoD, a master regulator of differentiation (Weintraub *et al.*, 1989; Halevy *et al.*, 1995; Guo *et al.*, 1995; Deato *et al.*, 2008). The results obtained so far demonstrate sustained level of cyclin D1 and delay in the expression of myogenin and p21 in CRYAB-C2C12 cells. The delay in the expression of these two important muscle differentiation regulatory proteins may be attributed to the impaired regulation of MyoD. To investigate the possible involvement of αB-crystallin in MyoD regulation, both C2C12 and CRYAB-C2C12 myoblasts were allowed to differentiate and MyoD expression profiles were monitored during the time course of differentiation. Our western blot analysis shows that in C2C12 myoblasts, the level of MyoD increases till 24 h of differentiation and decreases thereafter (Figure 3.7A and 3.7B). In contrast to C2C12 myoblasts cells, the expression of MyoD in CRYAB-C2C12 cells reaches a maximum at 36 h of differentiation, followed by sharp decrease in its level at later time points (Figure 3.7A and 3.7B). Thus, there is a shift in the maxima of MyoD expression by 12 h in CRYAB-C2C12 cells as compared to that of C2C12 cells. It is important to note that in C2C12 cells, MyoD was detected even at 0 h of differentiation while it was faintly detectable in CRYAB-C2C12 cells at similar time points.

To understand the mechanism by which αB-crystallin alters MyoD level, we sought to investigate whether the improper expression profile of MyoD is because of alteration in the protein synthesis or increased degradation or both. MyoD is a short-lived protein and gets degraded by the 26S proteasome pathway; hence inhibiting degradation will lead to its accumulation (Abu Hatoum *et al.*, 1998). C2C12 and CRYAB-C2C12 myoblasts were allowed to differentiate for 12 h and 24 h respectively and then treated with MG132 (10 μM),

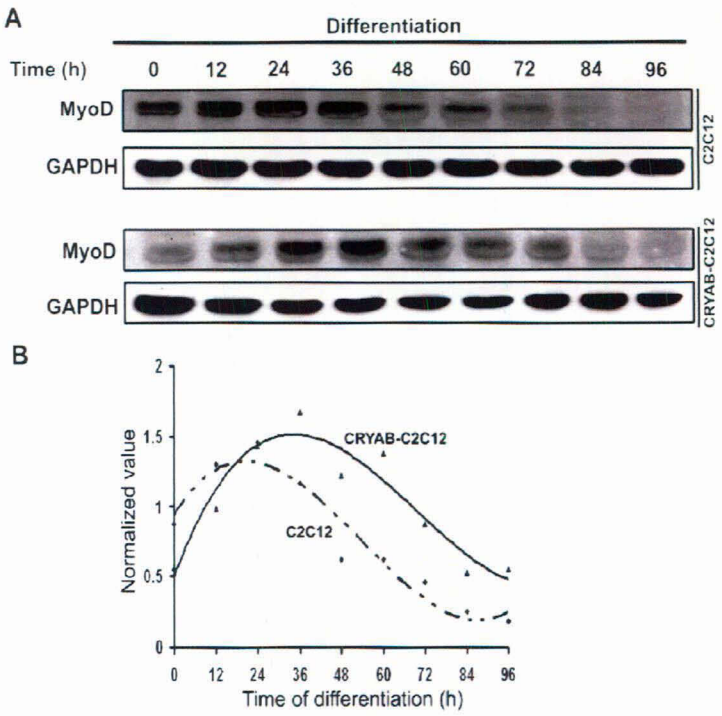


Figure 3.7. MyoD profile in C2C12 and CRYAB-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 cells were induced to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** show the western blots for the expression profile of MyoD of C2C12 and cells overexpressing αB-crystallin (CRYAB-C2C12). Western blot for GAPDH, used as loading control is also shown. **Panel B** shows the graphic representation of normalized band intensities from C2C12 (◆) and CRYAB-C2C12 (▲) cells.

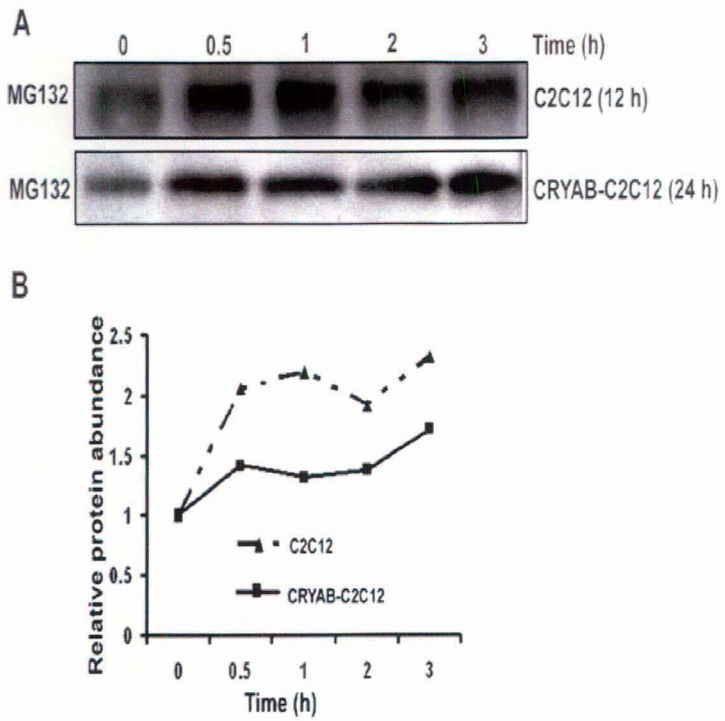


Figure 3.8. Relative MyoD accumulation in C2C12 and CRYAB-C2C12 cells. For determining the protein synthesis rate, C2C12 and CRYAB-C2C12 myoblasts were allowed to differentiate for 12 h and 24 h respectively. Subsequently, cells were treated with MG132 for 0, 0.5, 1, 2, 3 h, harvested and were evaluated for MyoD. **Panel A** shows the western blot for the accumulation of MyoD protein. **Panel B** describes the relative rate of synthesis of MyoD from C2C12 (▲) and CRYAB-C2C12 (■) cell lysates. The pixels for each band were measured and normalized so that the number of pixels at *t*=0 was 1. The pixels of each band were plotted *versus* time.

a potent inhibitor of the 26S proteasome. Upon treatment with MG132, MyoD accumulates in C2C12 as well as in CRYAB-C2C12 cells (Figure 3.8A and 3.8 B) with increasing time of incubation. However, the relative MyoD accumulation was approximately 2 fold higher in C2C12 myoblasts compared to that in CRYAB-C2C12 cells (Figure 3.8B). Thus, our data suggest that the shift in the profile of MyoD during differentiation in CRYAB-C2C12 cells could be due to its altered synthesis.

3.3.5 αB-crystallin enhances the degradation of MyoD

To determine whether the alteration in the MyoD expression is associated with modulation of its degradation, we compared the apparent half-life of endogenous MyoD protein in both C2C12 and CRYAB-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 myoblasts were incubated in differentiating medium (DM) for 24 hrs and then treated either with 50 μg/ml of cycloheximide (CHX), a protein synthesis inhibitor, alone or in combination with MG132 (10 μM). It was found that treatment of cells with CHX alone leads to decrease in MyoD level, whereas upon treatment of the cells with both CHX and MG132, the level of MyoD remains almost unchanged (Figure 3.9A and 3.9B).

It is important to note that in C2C12 cells, a significant level of MyoD was detected till 1 h of incubation with CHX alone, whereas there was a decrease in its band intensity in CRYAB-C2C12 cells at the same time point. As shown in Figure 3.9C and 3.9D, MyoD gets degraded in C2C12 cells with apparent half-life of 2.37 h ($t_{1/2} \approx 2.37$ h). Interestingly, in CRYAB-C2C12 cells, the degradation of MyoD was significantly accelerated with apparent half-life being 1.42 h ($t_{1/2} \approx 1.42$ h).

Upon treating the cells with CHX and MG132 together, we find that MyoD protein level is stabilized; indicating that the ubiquitin-proteasome pathway is responsible for the accelerated MyoD degradation in CRYAB-C2C12 cells (Figure 3.9). We also compared the apparent half-life of αB-crystallin during differentiation, and it seems to be highly stable in C2C12 cells as well as in CRYAB-C2C12 cells (Figure 3.10).

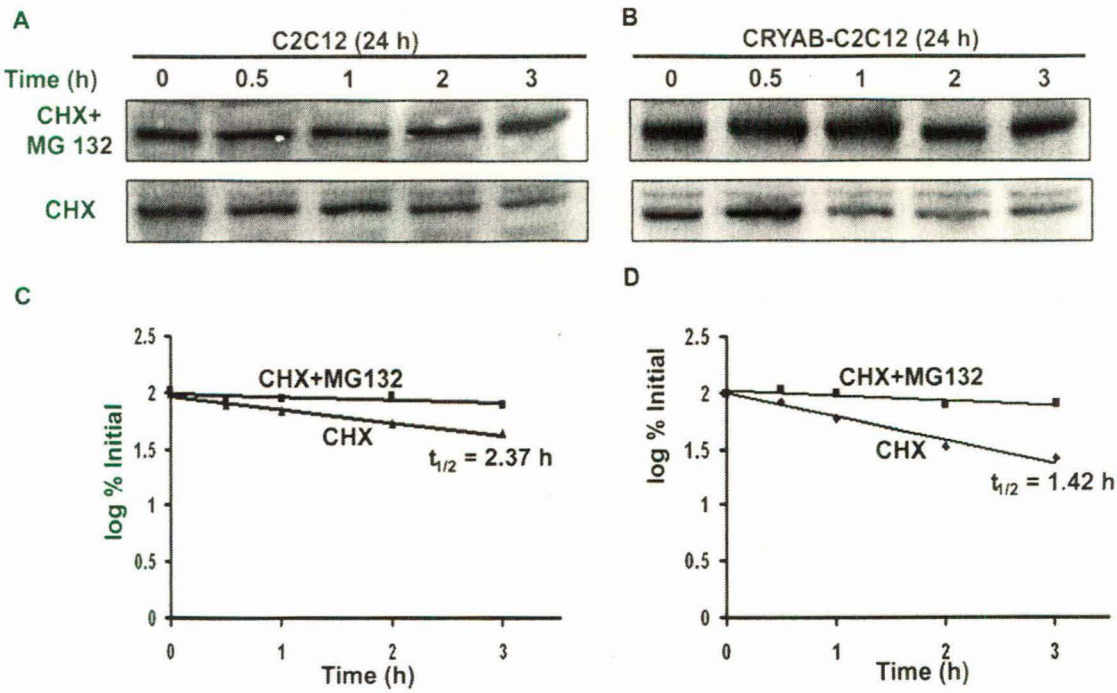


Figure 3.9. Apparent Half-life of endogenous MyoD in C2C12 and CRYAB-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 cells were induced for differentiation till 24 h of time. Further, cells were treated with CHX or CHX plus MG132. Cells were harvested, lysed at 0, 0.5, 1, 2, and 3 h and subjected to SDS-PAGE followed by western blot for MyoD protein (**Panel A and B**). An equal volume of each lysate was loaded onto the gel. The pixels for each band were measured and normalized so that the number of pixels at $t = 0$ was 100%. The $\log_{10}$ of the percentage of pixels was plotted *versus* time, and the $t_{1/2}$ was calculated from the $\log_{10}$ of 50% (**Panel C and D**).

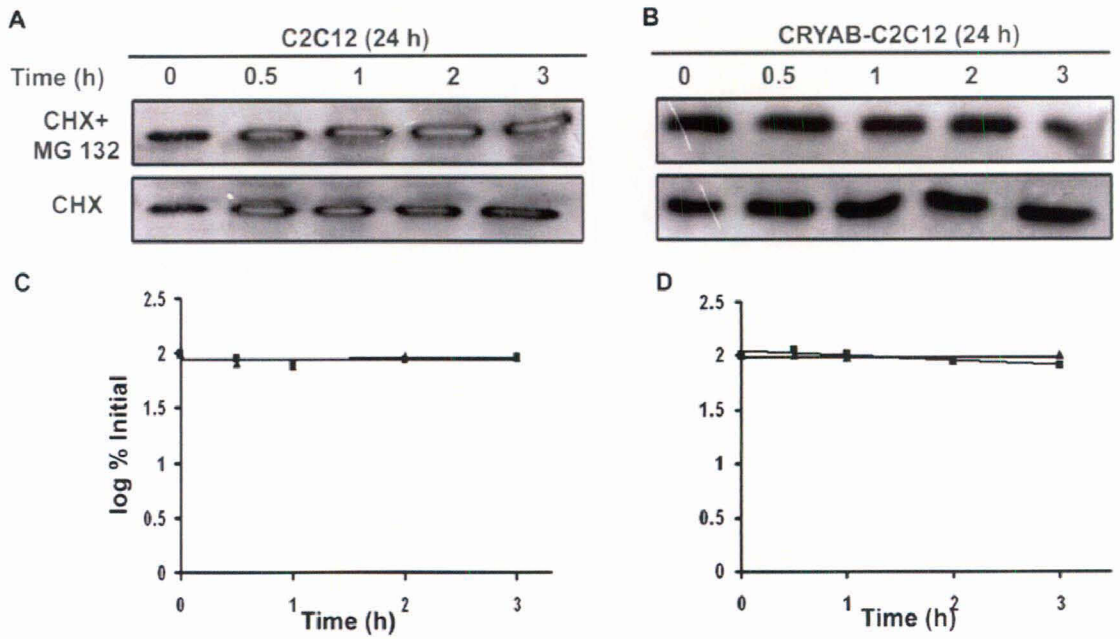


Figure 3.10. Half-life of αB-crystallin in C2C12 and CRYAb-C2C12 cells during differentiation. C2C12 and CRYAB-C2C12 cells were induced for differentiation till 24 h of time. Further, cells were treated with CHX alone or with CHX and MG132. Cells were harvested, lysed at 0, 0.5, 1, 2, and 3 h and subjected to SDS-PAGE and western blot for αB-crystallin protein (**Panel A and B**). An equal volume of each lysate was loaded onto the gel. The pixels for each band were measured and normalized so that the number of pixels at $t = 0$ was 100%. The $\log_{10}$ of the percentage of pixels was plotted *versus* time, and the $t_{1/2}$ was calculated from the $\log_{10}$ of 50% (**Panel C and D**).

To further elucidate the mechanism of the accelerated degradation of MyoD in CRYAB-C2C12 cells, we performed immuno-precipitation experiments using Ub-antibody at different time points after induction of differentiation. Our immunoblot analysis of the immuno-precipitated complex shows MyoD protein band in C2C12 cells as well as in CRYAB-C2C12 cells. Importantly, the immuno-precipitated complex consists of MyoD of higher molecular weight compared to normal molecular weight of MyoD (40 kDa). This can be attributed to the ubiquitination of MyoD during the differentiation process. Further, the extent of ubiquitination seems to increase with increasing time of differentiation. Our results show that in C2C12 cells, no ubiquitinated band of MyoD was found initially upon differentiation and at 12 h of differentiation. The detectable MyoD band was observed at 36 h of differentiation, which then increased at later time points and could be prominently visualized at 60 h of differentiation (Figure 3.11).

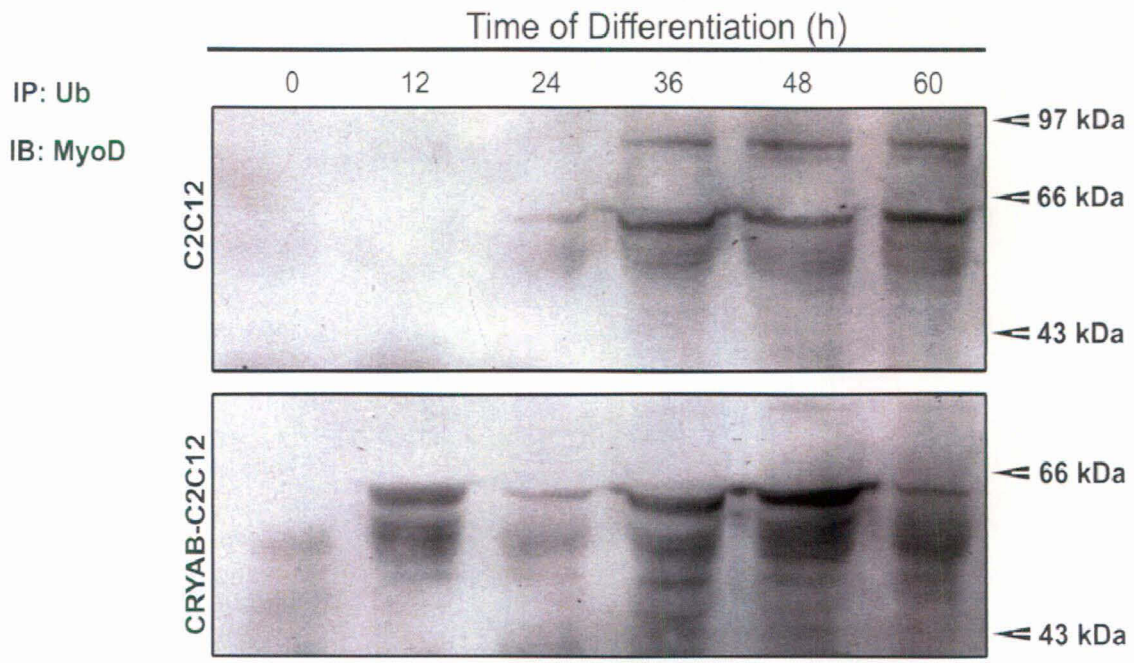


Figure 3.11. Immunoblot showing the extent of Ubiquitination during C2C12 and CRYAB-C2C12 differentiation. C2C12 and CRYAB-C2C12 myoblasts were allowed to differentiate in differentiating medium (DM) for different length of time. Cells were harvested and total cell extract was incubated with rabbit polyclonal anti-Ubiquitin antibodies at 4 °C for 8 h. Subsequently, they were incubated with protein-G magnetic beads for 4 h at 4 °C. The proteins from the immuno-precipitated complexes were separated by SDS-PAGE (12% polyacrylamide gel) and transferred onto Nitrocellulose membrane. The membranes were immunoblotted with monoclonal MyoD antibodies. The image obtained was processed using Adobe photoshop (6.0)

Interestingly, we find that in CRYAB-C2C12 cells, the ubiquitinated MyoD band was detected at as early as 12 h of differentiation, followed by its gradual increase till 48 h of differentiation. After 60 hrs of differentiation, a significant reduction in the levels of ubiquitinated MyoD was observed in CRYAB-C2C12 cells (Figure 3.11). The marked decrease in the levels of ubiquitinylated-MyoD at 60 h after differentiation is suggestive of decreased level of MyoD due to enhanced degradation in CRYAB-C2C12 cells (Figure 3.11). Taken together, our data show that over expression of αB-crystallin leads to decreased synthesis and enhanced proteasomal degradation of MyoD.

3.3.6 Muscle differentiation induces αB-crystallin up-regulation and its phosphorylation

In order to study the effect of muscle differentiation on the expression of αB-crystallin, C2C12 and CRYAB-C2C12 cells were allowed to differentiate for different time points and cell lysates were probed with antibodies specific for αB-crystallin. Phosphorylation of αB-crystallin plays an important role in its localization, interaction with other proteins and modulation of its activity (Morrison *et al.*, 2003; den Engelsman *et al.*, 2004; den Engelsman *et al.*, 2005; Ahmad *et al.*, 2008). αB-crystallin has been shown to undergo phosphorylation at Ser-59 and Ser-45 residues under stress conditions such as hyperthermia and oxidative stress, by p38-MAP kinase and p44-MAP kinase respectively (Ito *et al.*, 1997; Kato *et al.*, 1998). We have investigated the expression profile as well as the phosphorylation status of αB-crystallin using specific antibodies at different time points of differentiation. In agreement with an earlier report (Ito *et al.*, 2001a), our western blot analysis shows a gradual increase in the level of αB-crystallin in C2C12 cells upon differentiation (Figure 3.12A). A similar gradual increase in the expression of αB-crystallin was also found in CRYAB-C2C12 cells.

Using antibodies specific for Ser-59-phosphorylated αB-crystallin, we observed that the level of Ser-59-phosphorylated αB-crystallin increases gradually with increasing time of differentiation in both C2C12 and CRYAB-C2C12 cells. On the other hand, using antibodies specific for Ser-45-phosphorylated αB-crystallin could not detect any phosphorylation (data not shown).

Since p38-MAPK is known to phosphorylate Ser-59 residue in αB-crystallin, we investigated the presence of activated form of p38-MAP kinase (phospho-p38-MAPK) during the differentiation process. Our western blot data show that the level of p-p38 MAPK increases with increasing time of differentiation (Figure 3.12B) in both C2C12 and CRYAB-C2C12 cells.

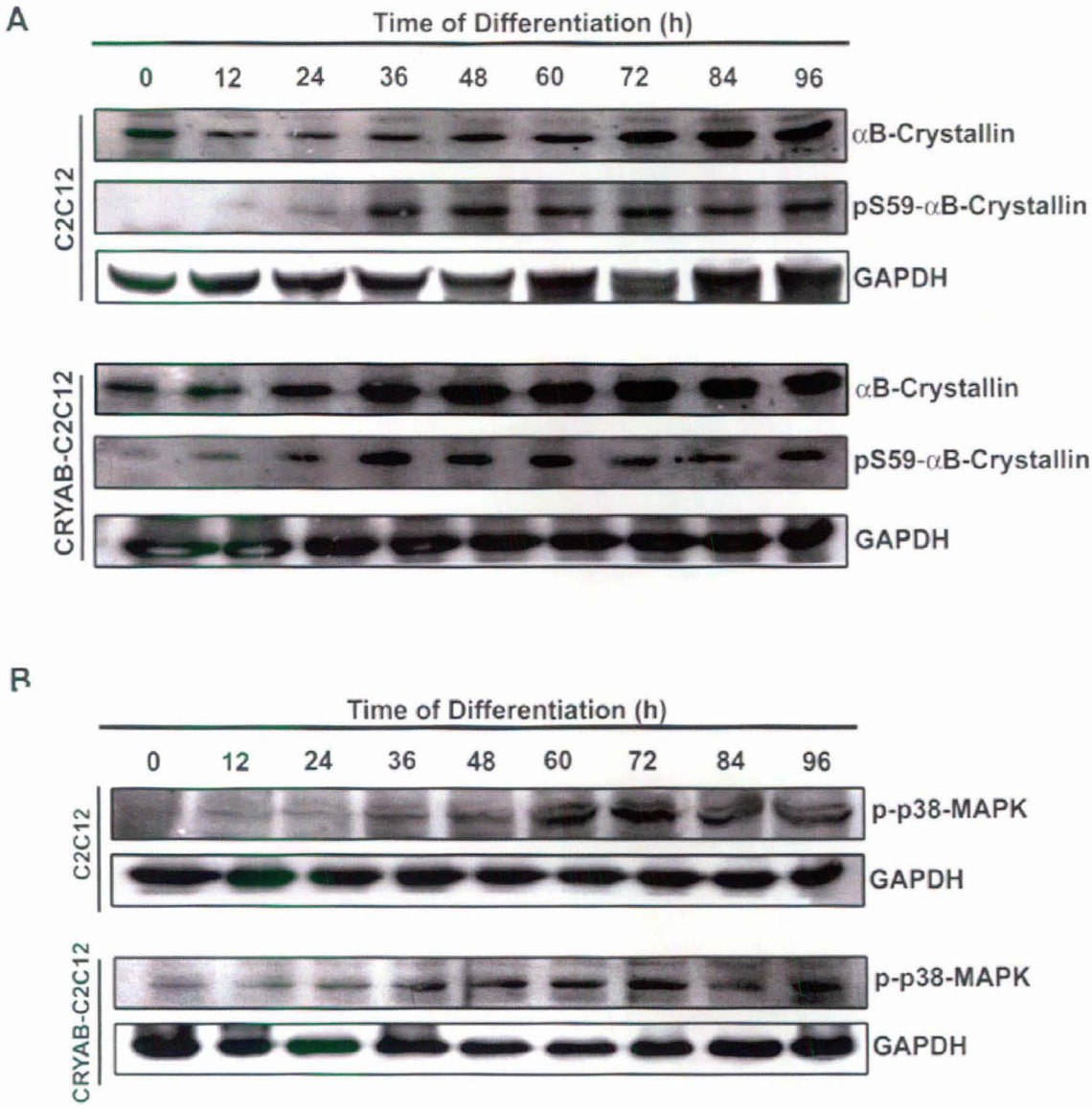


Figure 3.12. Western blots showing p-p38-MAPK, αB-crystallin and phospho-Ser-59-αB-crystallin profile during C2C12 and CRYAB-C2C12 cells differentiation. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** shows the western blot of αB-crystallin and phospho-Ser-59-αB-crystallin profile. **Panel B** shows the profile of phospho-p38 MAPK from C2C12 and cells overexpressing αB-crystallin (CRYAB-C2C12) lysates.

The increase in the expression or activation of p38-MAPK during the differentiation process seems to be essential, as inhibition of its activity using specific inhibitors leads to abrogation of the myogenesis process (Cuenda and Cohen, 1999; Wu *et al.*, 2000). Taken together, our data shows that the expression of αB-crystallin as well as its phosphorylation

at Ser-59 position increases during muscle differentiation (Figure 3.12) which may be critical for the process of differentiation of myoblasts.

Further, we performed immunostaining to analyze the localization of αB-crystallin by confocal microscopy in C2C12 and CRYAB-C2C12 cells during the differentiation process. We find that in myoblasts, αB-crystallin is predominantly localized in the cytoplasm with a some speckle-like staining in the nucleus in both C2C12 and CRYAB-C2C12 cells (Figure 3.13). Importantly, over-expression of αB-crystallin does not result in altered localization in CRYAB-C2C12 cells. It was observed that the fluorescence intensity of αB-crystallin staining gradually increased with increasing time of differentiation, indicating its enhanced expression during the differentiation process. It is important to note that unlike in myoblasts, the nuclei of the differentiated myotubes were devoid of speckle-like staining; αB-crystallin remained localized exclusively to the cytoplasm of the myotubes (Figure 3.13).

Importantly, confocal images of basal optical sections show that αB-crystallin exhibits fiber-like morphology in the myotubes of C2C12 as well as CRYAB-C2C12 cells (Figure 3.14). The fiber-like appearance of αB-crystallin was not observed in the myoblasts stage, suggesting a specific role for αB-crystallin at later stages of differentiation. Since, extensive reorganization of cytoskeleton takes place during the differentiation process, it may be speculated that αB-crystallin is associated with the modulation of cytoskeleton and/or associated proteins.

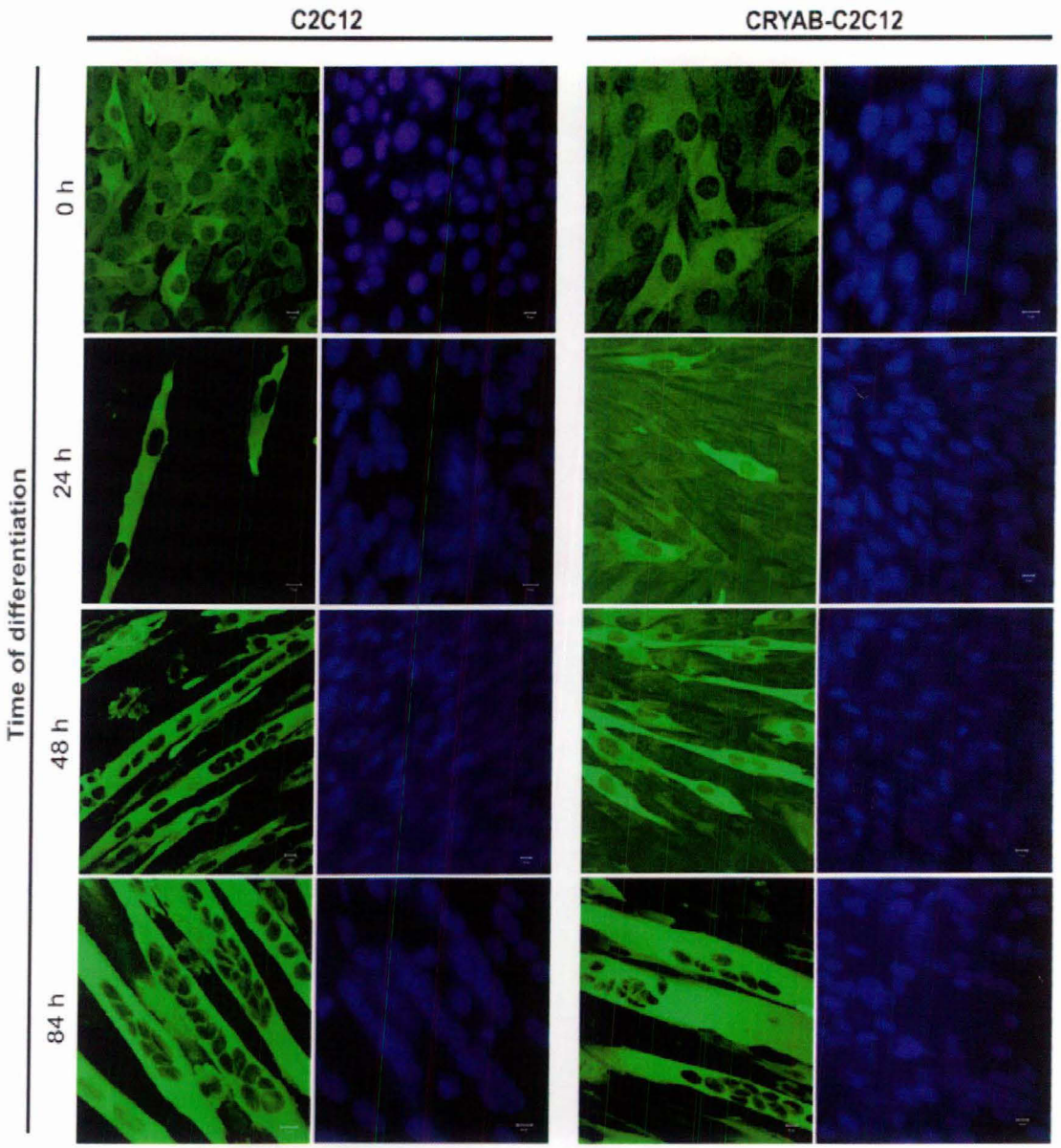


Figure 3.13. Immunolocalization of αB-crystallin during differentiation. C2C12 and CRYAB-C2C12 myoblasts, grown on coverslips were induced to differentiate for different length of time. The cells were fixed in 3.7% (v/v) formaldehyde and immunostained using αB-crystallin-specific antibodies. The nuclei were counterstained with DAPI. The scale bar represents 10 μm.

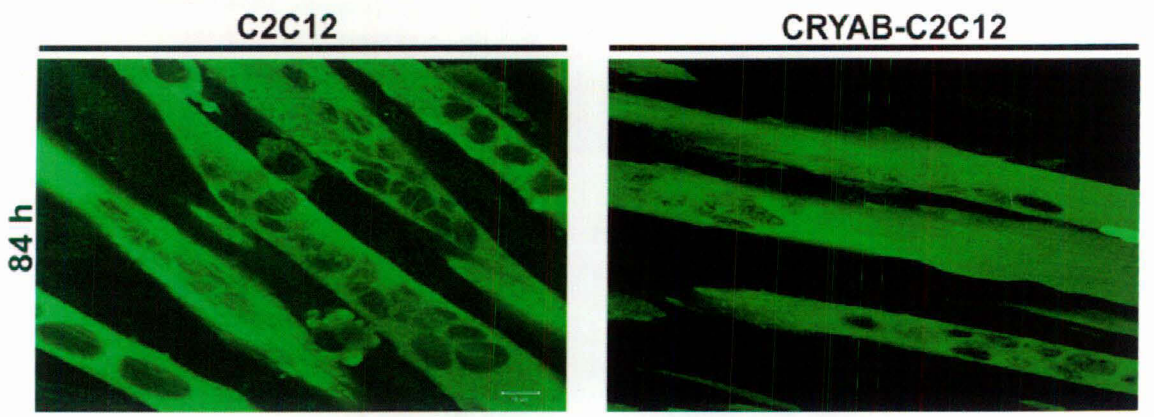


Figure 3.14. Immunolocalization of αB-crystallin at the basal region during differentiation. C2C12 and CRYAB-C2C12 myoblasts, grown on coverslips were induced to differentiate for different length of time. The cells were fixed in 3.7% (v/v) formaldehyde and immunostained using αB-crystallin-specific antibodies. The nuclei were counterstained with DAPI. The confocal images were obtained with optical sections of 0.3 μm. The scale bar represents 10 μm.

3.3.7 αB-crystallin inhibits caspase-3 activation during differentiation

During skeletal muscle differentiation, a subset of myogenic precursor cells acquires apoptotic resistance and is destined to undergo terminal differentiation (Walsh, 1997). Caspase-3 activation seems to be essential for skeletal muscle differentiation as it is shown that knockout of *caspase-3* leads to inhibition of muscle differentiation (Fernando *et al.*, 2002). Various reports demonstrate that caspase-3 activity is responsible for the apoptotic cell death during muscle differentiation (Mukasa *et al.*, 1999). Therefore, a proper modulation of caspase-3 activity is essential for myogenic differentiation. We set out to examine the effect of αB-crystallin on the activation of caspase-3 during the differentiation of C2C12 and CRYAB-C2C12 myoblasts. It is known that pro-caspase-3 is processively cleaved to pro-active caspase-3 (p24), which subsequently gets cleaved to active caspase-3 (p17/12) (Nunez *et al.*, 1998; Boatright *et al.*, 2003). Our western blot analysis shows that pro-caspase-3 gets activated and cleaved to p17/p12 (active caspase-3) in the C2C12 cells (Figure 3.15). Moreover, there was increasing accumulation of pro-active caspase-3 (p24) along with p17/12 fragments (active caspase-3) at later stages of differentiation in C2C12 cells. Interestingly, we find that in CRYAB-C2C12 cells, the cleavage of pro-caspase-3 was restricted to pro-active caspase-3 (p24), as there was increasing accumulation of pro-active caspase-3 (p24) with increasing time of differentiation (Figure 3.14).

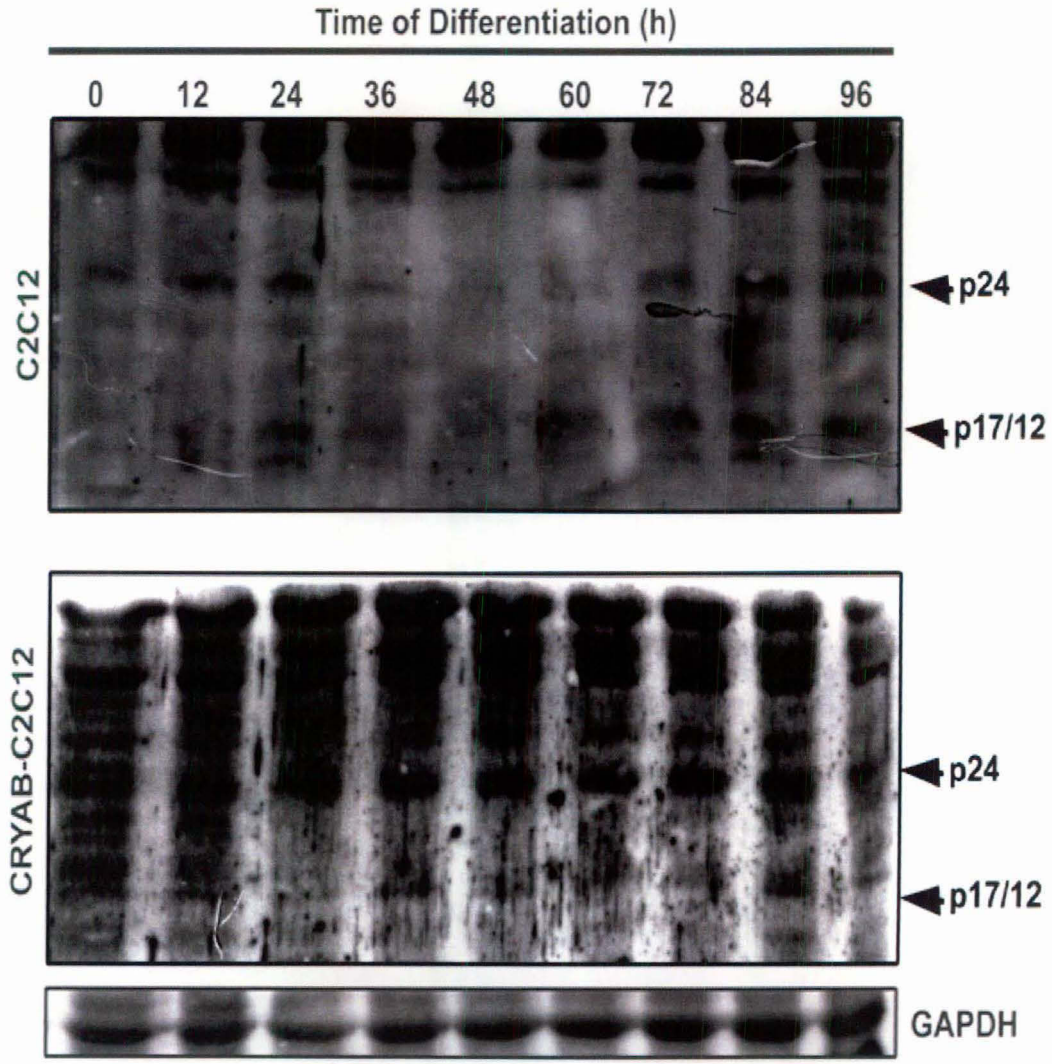


Figure 3.15. Western blots showing caspase 3 profile during C2C12 and CRYAB-C2C12 cells differentiation. C2C12 and CRYAB-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. Western blot analysis for caspase-3 cleavage was perfomed using C2C12 and CRYAB-C2C12 cell lysates. The arrows indicate the cleaved products from pro-caspase-3 to form pro-active caspase-3 (p24) and active caspase-3 (p17/12).

These data suggest that αB-crystallin inhibits caspase-3 activity by preventing the maturation of pro-caspase-3 during muscle differentiation.

3.4 Discussion

Small heat shock proteins (sHSPs) are constitutively expressed in several tissues including skeletal muscles, where they have been shown to be involved in stress tolerance and muscle maintenance (Neufer and Benjamin, 1996; Sugiyama *et al.*, 2000). Skeletal muscles are constantly subjected to stress or injury and require repair and regeneration to maintain tissue homeostasis. It is important to understand the biology of muscle regeneration for the development of effective therapeutic intervention as and when necessary. During the muscle regeneration process, a sub-set of cells, called as satellite cells gets stimulated to proliferate followed by their exit from the cell cycle to engage in the myogenic differentiation program (Hawke and Garry, 2001).

Skeletal muscle differentiation is a dynamic process that involves sequential expression of myogenic regulatory factors (MRFs), essential for the proper myogenesis (Weintraub, 1993). During muscle differentiation, myoblasts undergo cell-cycle arrest and finally differentiate to form mature myotubes (Maione and Amati, 1997; Walsh and Perlman, 1997). Myogenic differentiation is under the strict control of myogenic transcription factors such as MyoD, Myf5, myogenin and MRF4 in co-ordination with a second class of transcription factors including MEF2A-2D (Molkentin *et al.*, 1995; Sartorelli and Caretti, 2005). It has been shown that mice lacking MyoD develop normally but are severely impaired in their ability to regenerate muscle after tissue injury (Yablonka-Reuveni *et al.*, 1999; Cooper *et al.*, 1999). MyoD is found to be a master regulator of muscle differentiation and is involved in the determination of the muscle cell lineage (Rudnicki *et al.*, 1993). Muscle appears to be unique tissue where as many as six out of ten mammalian sHSPs are expressed (Sugiyama *et al.*, 2000). A recent study by Sugiyama *et al.*, 2000, has shown that they form two different complexes during muscle differentiation, implying the importance of these sHSPs in muscle development. Of all the sHSPs present in the muscle, αB-crystallin seems to be very critical as it is shown that mice lacking αB-crystallin have lower muscle mass (Brady *et al.*, 2001). During muscle differentiation, the level of αB-crystallin goes up by 10-fold, further corroborating the significance of this protein in differentiation (Ito *et al.*, 2001a). A point mutation in αB-crystallin, R120G has been found to be associated with desmin related myopathies, suggesting its importance in muscle tissue homeostasis (Vicart *et al.*, 1998; Sanbe *et al.*, 2004). In spite of all these reports, the exact function of αB-crystallin during muscle development is not yet clearly understood.

In this study, we have probed the role of αB-crystallin and sequential events in muscle differentiation process. We have demonstrated that αB-crystallin modulates MyoD activity by the combined effect of reduced synthesis and increased degradation, thus delaying muscle differentiation. We find that the formation of myotubes is severely affected in cells over-expressing αB-crystallin (CRYAB-C2C12 cells) (Figure 3.1 and Figure 3.2). As it is well established that exit from the cell-cycle and differentiation are inter-linked, alteration in cell cycle may affect the differentiation. Cell-cycle analysis showed that CRYAB-C2C12 cells were more proliferative; the level of the critical cell cycle regulator, cyclin D1, is maintained for a longer period of time (Figure 3.5B and 3.5C). We also find that the expression of p21, a cell-cycle inhibitor, is delayed significantly in CRYAB-C2C12 cells compared to that in C2C12 cells (Figure 3.5). These results suggest that the cell-cycle process in CRYAB-C2C12 is continued for an extended period of time, which may contribute to the delay in the differentiation process. It seems likely that the increased expression of cyclin D1 is because of the enhanced activation of NF-κB in CRYAB-C2C12 cells. We have shown that αB-crystallin activates NF-κB in a phosphorylation-dependent manner and protects cells from TNF-α induced cytotoxicity (unpublished data). Moreover, activated NF-κB is known induce loss of MyoD mRNA (Guttridge *et al.*, 2000), thereby inhibiting skeletal muscle differentiation. Our results show that the maxima of MyoD expression gets shifted by about 12 h in CRYAB-C2C12 cells compared to the C2C12 cells (Figure 3.7A and 3.7B). Importantly, upon treatment with MG132, a 26S proteasome inhibitor, there was approximately 2 fold increased accumulation of MyoD in C2C12 cells than in CRYAB-C2C12 cells, suggesting that the rate of synthesis is slower in CRYAB-C2C12 cells (Figure 3.8). This may be due to increased activation of NF-κB, leading to MyoD mRNA loss in CRYAB-C2C12 cells. Further, we find that the apparent half-life of MyoD is reduced ($t_{1/2}$= 1.42 hrs) in CRYAB-C2C12 cells compared to normal C2C12 cells ($t_{1/2}$= 2.37 hrs) (Figure 3.9). Our immuno-precipitation experiments suggest that the accelerated degradation of MyoD is due to the enhanced ubiquitination in CRYAB-C2C12 cells compared to that in C2C12 cells (Figure 3.11). It is important to mention that αB-crystallin has been shown to be a part of the FBX4-Ub complex and promotes FBX4-dependent ubiquitination (den Engelsman *et al.*, 2003; Lin *et al.*, 2006). This enhanced ubiquitination is shown to be dependent on the phosphorylation of αB-crystallin at Ser-45 and Ser-19 residue (den Engelsman *et al.*, 2004). During the differentiation process, we show that αB-crystallin gets phosphorylated at Ser-59

residue (Figure 3.12). Whether this preferential phosphorylation plays a role in MyoD ubiquitination is not clear at present. Further experiments are needed to investigate the effect of phosphorylation during myogenic differentiation. During the cell division process, αB-crystallin gets phosphorylated at Ser-45 and Ser-19 residues and is shown to be involved in cyclin D1 ubiquitination (Kato *et al.*, 1998; Lin et al., 2006). It would be interesting to dissect out the phosphorylation-dependent function of αB-crystallin during cyclin D1 modulation and cell cycle progression. Contrary to the observation made earlier (Lin et al., 2006) we observed sustained levels of cyclin D1 during differentiation in cells over-expressing αB-crystallin. Our immuno-staining data shows that αB-crystallin remains exclusively in the cytoplasm of myotubes upon differentiation, unlike the speckle-like staining observed in the nucleus of myoblasts (Figure 3.13). Earlier studies from our laboratory, demonstrated that αB-crystallin co-localizes with the lamin A/C network in heat-stressed myoblasts but not in myotubes (Adhikari *et al.*, 2004). This assumes greater significance considering earlier findings of a reorganization of lamin A/C speckles to a diffuse network after differentiation of C2C12 myoblasts to myotubes. Further, our western blot using Ser-59 phospho-specific antibodies shows that the phosphorylation of αB-crystallin increases with increasing time of differentiation. We also observed that the level of p38-MAP kinase increases gradually with induction of differentiation (Figure 3.12B). This observation is consistent with the earlier report of activation of p38-MAP kinase during the differentiation process (Gredinger *et al.*, 1998): p38-MAP kinase is involved in phosphorylation of Ser-59 in αB-crystallin.

During the differentiation process, one of the critical factors is the ability to survive under differentiating conditions. αB-crystallin has been shown to negatively regulate apoptosis by inhibiting caspase-3 activation during differentiation (Kamradt *et al.*, 2002). Interestingly, caspase-3 activity is required for the skeletal muscle differentiation, as it is involved in the cleavage of regulatory proteins important for myogenesis (Fernando *et al.*, 2002). Thus critical balance of activation and inhibition caspase-3 appears to be important. We show that αB-crystallin prevents the activation of caspase-3 (Figure 3.15), which may contribute towards the delay in the muscle differentiation process. Taken together, our data suggest that αB-crystallin is essential for muscle differentiation and regulation of αB-crystallin is very important for proper myogenesis.

CHAPTER 4

Effect of R120G-αB-crystallin mutant on muscle differentiation

4.1 Introduction

Muscle wasting is a disease that leads to extensive loss of muscle fibers. Muscle wastage has been found to be associated with many chronic wasting syndromes such as AIDS, chronic heart failure and chronic obstructive pulmonary disease (COPD) (Schols *et al.,* 1998; Kotler *et al.,* 1989; Anker *et al.,* 1997). Multiple lines of evidence suggest that imbalances in processes that govern the maintenance of skeletal muscle and muscle plasticity, such as skeletal muscle fiber regeneration, maintenance and apoptosis, may be an important determinant of muscle wasting (Amack and Mahadevan, 2004; Langen *et al.,* 2004). Alternatively, muscle wasting is also believed to result from disturbances in the energy or protein anabolism-catabolism balance. For example, imbalances in myofibrillar protein synthesis and proteolysis have been demonstrated in experimental models of cancer cachexia or sepsis (Buck and Chojkier, 1996; Vary *et al.,* 1996). Mutations in the myofibrillar proteins such as desmin, filamin C and myotilin have been shown to be associated with myopathies (Goldfarb *et al.,* 1998; Selcen, 2008). Despite the increasing genetic heterogeneity, the clinical and morphologic phenotypes are remarkably similar in myofibrillar myopathies.

Diseases such as myopathies and neuropathies are characterized by intracellular protein aggregates, which have been implicated in inducing cell death (Maloyan *et al.,* 2005). Protein aggregation-induced skeletal myopathies and cardiomyopathies are shown to be caused by mutations in αB-crystallin (HSPB5) or desmin and are characterized by protein misfolding and large cytoplasmic aggregates (Munoz-Marmol *et al.,* 1998; Vicart *et al.,* 1998; Dalakas *et al.,* 2000; Wang *et al.,* 2001). Alteration or modulation of the assembly of desmin due to its mutations has been implicated in onset of desmin related myopathy (DRM) (Goldfarb *et al.,* 2004). Importantly, αB-crystallin, a molecular chaperone, has been shown to be involved in the modulation of intermediate filament assembly (Nicholl and Quinlan, 1994). The compromised assembly of desmin mutants is found to be associated with severe familial cardiac and skeletal myopathies (Goebel, 1995; Munoz-Marmol *et al.,* 1998). DRMs are inherited neuromuscular disorders that are characterized by the accumulation of aggregates containing desmin and αB-crystallin (Wang *et al.,* 2001; Goebel, 2003). In skeletal myofibrils and cultured cardiomyocytes, αB-crystallin is colocalized with desmin at the Z-bands (Bennardini *et al.,* 1992). It is found that αB-crystallin stabilizes and

protects target proteins including desmin by preventing their irreversible aggregation (Perng *et al.,* 1999; Chávez Zobel *et al.,* 2003). These reports suggest that αB-crystallin plays an important role in the maintenance of cytoskeletal proteins and modulates their dynamics. It is important to note that a point mutation in αB-crystallin (R120G-αB-crystallin), is shown to be the cause of an autosomal-dominant desmin-related myopathy (DRM), also called as αB-crystallinopathy (Vicart *et al.,* 1998; Goebel and Warlo, 2000). Studies from our laboratory as well as other simultaneous independent studies have shown that the R120G-αB-crystallin has altered structure and significantly reduced chaperone activity implicating the loss of chaperone activity to be the molecular basis for DRM (Kumar *et al.,* 1999; Perng *et al.,* 1999; Bova *et al.,* 1999). It was suggested that, in α-crystallinopathy, desmin collapses with R120G-αB-crystallin due to a reduced chaperone activity of R120G-αB-crystallin (Perng *et al.,* 1999; Perng *et al.,* 2004). Chaperone activity of αB-crystallin is found to be necessary for the proper organization of the desmin filaments (Nicholl and Quinlan, 1994). Desmin is the first muscle-specific cytoskeletal protein to be expressed during myogenesis (Li and Capetanaki, 1993). It has been shown by knockout studies that reducing the expression of desmin lead to complete loss of myoblasts fusion, which gets rescued by over-expression of desmin (Capetanaki *et al.,* 1997). Taken together, these reports suggest that desmin is very critical for myogenesis as well as for muscle homeostasis.

The involvement of αB-crystallin in muscle differentiation and its ability to modulate intermediate filament assembly seems to be highly significant in muscle maintenance and homeostasis. However, the role of disease causing R120G mutant of αB-crystallin in the process of muscle differentiation is not investigated. It is not clear which of the two, loss of chaperone activity or the toxic interaction of R120G-αB-crystallin with desmin is responsible for the alteratered myogenesis? Interestingly, it has been shown that mice with desmin mutations as well as mice lacking desmin or αB-crystallin have much less severe myopathy than those expressing the R120G-αB-crystallin mutant (Wang *et al.,* 2001; Chavez Zobel *et al.,* 2003). This observation suggests that R120G-αB-crystallin induced myopathy might result from the toxic effects of the R120G-αB-crystallin rather than the loss of function of αB-crystallin or desmin.

In this chapter we have investigated the effect of R120G-αB-crystallin in the muscle differentiation process using C2C12 mouse myoblasts as a model system. We find that

differentiation is severely affected in cells over-expressing R120G-αB-crystallin. Our study suggests that the inhibition of the differentiation seems to be due to the cytotoxicity induced by R120G-αB-crystallin. Further, we have carried out experiments to decipher the effect of R120G-αB-crystallin in the differentiation pathway.

4.2 Materials and Methods

4.2.1 Antibodies and Reagents

DMEM, Fetal Calf Serum (FCS), DMSO, MTT, mouse monoclonal anti-Flag antibodies were purchased from (Sigma Chemical Company, USA). Cycloheximide, MG132, rabbit polyclonal anti-ubiquitin antibodies were obtained from Calbiochem, EMD Biosciences, Germany. Propidium Iodide (PI), Alexa-488- and Cy3-conjugated secondary antibodies were obtained from Molecular Probes, Invitrogen Corp. Oregon, USA. Anti-GAPDH mouse monoclonal antibodies were purchased from Chemicon International Inc., USA. Rabbit polyclonal antibodies for αB-crystallin, phosphoserine-59-αB-crystallin and phosphoserine-45-αB-crystallin and mouse monoclonal anti-cyclin D1 antibodies were obtained from Stressgen Biotechnologies, Victoria, Canada. Mouse monoclonal antibodies for MyoD were purchased from Dako Cytomation, USA; rabbit polyclonal anti-myogenin antibodies were from Santa Cruz, USA. Mouse monoclonal antibodies for p21 and phospho-p38 MAPK were from BD Transductions, Pharmingen, USA. Protease inhibitor cocktail was procured from Roche Applied Sciences, USA; Lipofectamine-2000 was obtained from Invitrogen, USA. HRPO-conjugated anti-rabbit, anti-mouse secondary antibodies and Enhanced Chemiluminescence (ECL) Western blot detection kit were purchased from Amersham Biosciences, USA. Vectashield mounting medium containing 4', 6-diamidino-2-phenylindole (DAPI) was from Vector Laboratories, USA.

4.2.2 Plasmids and Construction of FLAG-tagged cDNAs

The plasmid pCDNA 3 having Flag epitope upstream of the multiple cloning sites (MCS) was used as a control vector. cDNA of the full-length αB-crystallin-R120G was PCR-amplified using the following primers: **Forward primer-** 5'-

GGCCGAATTCATGGACATCGCCATCCACCAC-3'and **Reverse primer-** 5'-GCCCTCGAGCTATTTCTTGGGGGCTGCGG-3'. The PCR products were digested with EcoR I and Xho I restriction enzymes and ligated into a modified pCDNA3 vector, in frame with the FLAG epitope, inserted upstream of the MCS. The final positive clones were confirmed by automated DNA sequencing.

4.2.3 Cell culture and differentiation

C2C12, mouse skeletal myoblast cell line, was maintained at sub-confluent densities (60-70%) in DMEM supplemented with 20% fetal calf serum (FCS), 100 units/ml of penicillin and100 μg/ml of streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. To induce differentiation, cells at 89-90% confluence were shifted to DMEM supplemented with 2% horse serum, (differentiating medium (DM)). At different time-points of differentiation, cells were lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), containing 50 mM NaCl, 5 mM EDTA, 1 mM PMSF, 50 mM NaF, 0.2 mM Na_3VO_4, 0.2% (v/v) NP-40 and Protease Inhibitor Cocktail (PIC)). For immunofluorescence studies, cells were grown on coverslips and allowed to differentiate for different lengths of time, as indicated. Cells were fixed with 3.7% (v/v) formaldehyde after each time-point and processed for confocal analysis. The differentiation index (DI) and fusion index (FI) was calculated as described in the text.

4.2.4 Stable transfections

For stable transfections, C2C12 cells were grown in a six well plate and transfected either with 1μg pCDNA3 vector alone or with 1μg of pCDNA3-N FLAG-tagged-R120G-αB-crystallin plasmids using Lipofectamine 2000 reagent (Invitrogen Corp., USA). At 48h post-transfection, the cells were sub-cultured in medium containing geneticin (Roche Applied Sciences, USA) and grown for a period of one month. Subsequently, stably transfected clones of R120G-αB-crystallin were selected in the presence of 500 μg/ml of G418 (Geneticin) (Invitrogen Corp., USA). Single cell clones were isolated and the ones expressing not less than two fold higher levels of R120G-αB-crystallin (as determined by western blot analysis using anti-Flag antibodies) were expanded and used for our study. C2C12 cells that stably over-expressed R120G-αB-crystallin were referred to as R120G-C2C12.

4.2.5 Cell survival assay

C2C12 cells and cells over-expressing R120G-αB-crystallin (R120G-C2C12) were grown on cover slips till 80-90% confluency. Subsequently, cells were shifted to differentiating medium (DM) and allowed to undergo differentiation for different time periods. Cells were washed twice with PBS and incubated with MTT (50 µg/ml) for 4 hrs. DMSO was added to dissolve the formazan crystals and OD was taken at 570 nm. The graph was plotted as optical density (570 nm) versus time of differentiation.

4.2.6 FACS analysis

C2C12 cells and R120G-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. At each time point, cells were fixed in 80% methanol, stained with propidium iodide, and cell cycle analysis was done using Fluorescence Activated Cell Sorter, (Facs Caliber-Becton and Dickinson USA). The values represent average of three independent experiments and are expressed as percentage.

4.2.7 Immunofluorescence microscopy

C2C12 cells and cells over-expressing R120G-αB-crystallin (R120G-C2C12) were grown on cover slips till 80-90% confluency. Subsequently, cells were shifted to differentiating medium (DM) and allowed to undergo differentiation for different time periods. At each time point, cells were washed twice with ice-cold PBS and fixed with 3.7% formaldehyde. The fixed cells were permeabilized with 0.05% Triton X-100 for 8 min. After blocking with 2% BSA, cells were incubated with specific antibodies either for myogenin or αB-crystallin, followed by incubation with Alexa-488- and Cy3-tagged secondary antibodies respectively. The cells were mounted in Vectashield medium containing DAPI. Confocal laser scanning microscopy was performed on a Carl Zeiss inverted microscopy. Image analysis was done using LSM 510 meta software (Version 5).

4.2.8 SDS-PAGE and Western Blot analysis

C2C12 cells and R120G-C2C12 cells were induced to undergo differentiation in DM and harvested at respective time points. Cells were lysed in ice-cold lysis buffer, sonicated and centrifuged at 14000 rpm for 10 min at 4 °C. Equal amount of protein was subjected to SDS-PAGE (12% polyacrylamide gels) and transferred to nitrocellulose membrane using a semi-dry transfer apparatus (Amersham Pharmacia). The membrane was blocked with 10% (w/v) milk protein and incubated sequentially with appropriate primary antibodies and HRPO-conjugated secondary antibodies and was visualized using ECL kit (Amersham Biosciences, USA) according to the manufacturer's instructions. The band intensities were quantified by densitometry using GeneTools software (Syngene). The ratio of band intensities of the respective blots and that of the corresponding loading control, expressed in arbitrary units was used for comparison. The values represent an average of a minimum of three independent experiments.

4.2.9 Determination of Protein Degradation Half-life

As described above, C2C12 cells and R120G-C2C12 cells were induced to differentiate for 24 hrs, following which, cells were incubated with cycloheximide (CHX, 50 μg/ml, Sigma) to inhibit further protein synthesis. The 26S proteasomal inhibitor MG132 (N-benzyloxycarbonyl-Leu-Leu-leucinal, 10 μM, Calbiochem) was added along with CHX when necessary. Following incubation for 0, 0.5, 1, 2, and 3 hrs, cells were lysed in lysis buffer, and equal amounts of total cell protein were subjected to SDS-polyacrylamide gel electrophoresis. Western blotting was performed as described above using specific antibodies for MyoD. The band intensities were quantified using GeneTools software (Syngene), the graph was plotted using the EXCEL graphing program (Microsoft). The pixels for each band were measured and normalized so that the number of pixels at $t = 0$ was 100%. Protein degradation rate is expressed as apparent half-life ($t_{1/2}$), the time for degradation of 50% of the protein from its initial value. The $\log_{10}$ of the percentage of pixels was plotted *versus* time, and the $t_{1/2}$ was calculated from the $\log_{10}$ of 50%.

4.3 Results

4.3.1 R120G-αB-crystallin mutant inhibits myogenic differentiation

To investigate the effect of R120G-αB-crystallin during myogenic differentiation, normal C2C12 myoblasts and cells stably over-expressing R120G-αB-crystallin mutant (R120G-C2C12) were allowed to differentiate for different time periods and analyzed for the ability to undergo myogenic differentiation. The phase-contrast micrographs show that normal C2C12 myoblasts fused into myotubes and extensive myotube formation was observed at 72 h of differentiation. In comparison to normal C2C12 myoblasts, R120G-C2C12 cells showed significant cell death with no myotube formation at similar time points (Figure 4.1).

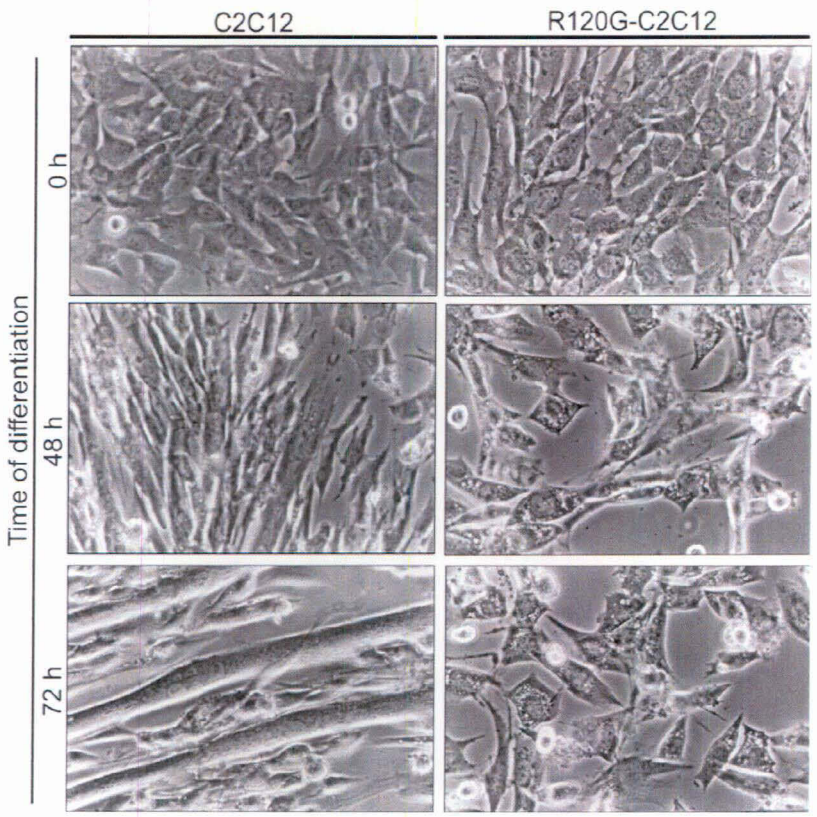


Figure 4.1: R120G-αB-crystallin inhibits myogenic differentiation. Normal C2C12 cells and cells over-expressing R120G-αB-crystallin (R120G-C2C12) were grown to 90-100% confluency in growth medium (GM). Subsequently, cells were allowed to differentiate in DMEM containing 2% horse serum (DM). Phase contrast images were taken at different time-points at a magnification of 200X using inverted microscope, Nikon, Japan. The images obtained were further processed using adobe photoshop version 6.0.

Further, unlike C2C12 cells, R120G-C2C12 cells showed formation of vacuole-like structures in the cytoplasm during the differentiation process. Recently, Tannous *et al.,* have demonstrated that autophagy, characterized by increased vacuole formation, is an adaptive response in desmin-related cardiomyopathy. To further investigate the differentiation process, we calculated the differentiation index (DI) to find out whether the differentiation process or the fusion of myoblasts was inhibited. The differentiation index was calculated as described earlier (see chapter III).

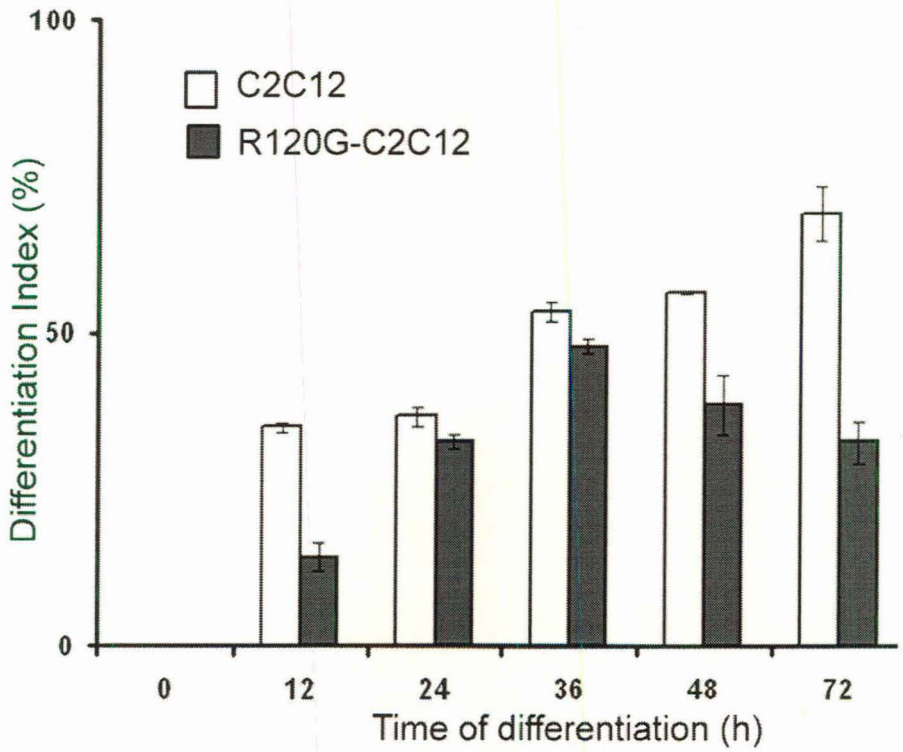


Figure 4.2: R120G-αB-crystallin mutant reduces differentiation index during differentiation. C2C12 and cells over-expressing R120G-αB-crystallin mutant (R120G-C2C12) were grown on coverslips to 90-100% confluency in growth medium (GM). Differentiation was induced by shifting the cells in DMEM medium containing 2% horse serum. Cells were fixed and immunostained using antibody specific to myogenin at various time-points. The DI was calculated based on the total number of myogenin positive cells versus total number of nuclei and represented as bar diagrams.

As shown in figure 4.2, at 12 h of differentiation, the DI of C2C12 cells were significantly higher than that of R120G-C2C12 cells. Further, we find that the DI of both

C2C12 cells and R120G-C2C12 cells become comparable at 36 h of differentiation. At later time points, the DI of C2C12 cells gradually increased to ~75% at 72 h of differentiation, whereas in R120G-C2C12 cells, it decreased sharply and was ~ 32% at 72 h of differentiation. The differentiation index profile (shown above) suggests that the differentiation program was initiated in R120G-C2C12 cells; however the differentiation of myoblasts appears to be severely affected at later time points. Importantly, we found no myotube formation in R120G-C2C12 cells at any stage of differentiation except few sac-like structures during the later time points of differentiation process.

We performed the cell survival assay to check if the inhibition in myotube formation was because of cell death during differentiation of R120G-C2C12 cells. We find that a large proportion of C2C12 cells were surviving where as in R120G-C2C12 cells, a significant number of cells were dying and the cell confluence was gradually decreasing as reflected by the decrease in the cell confluence (Figure 4.1).

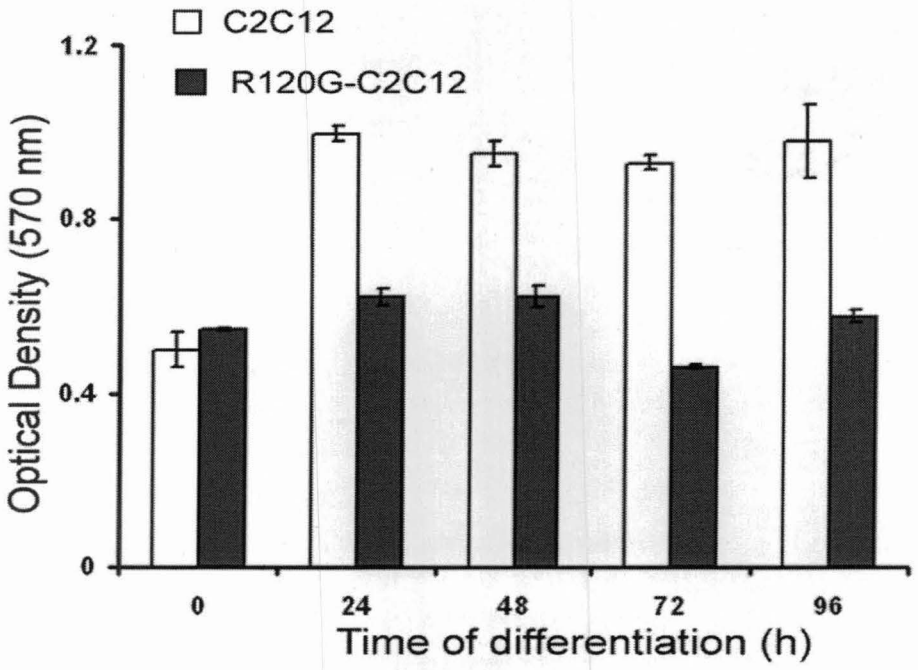


Figure 4.3: MTT assay for cell survival in C2C12 cells and R120G-C2C12 cells. C2C12 cells and R120G-C2C12 cells were allowed to differentiate in DMEM containing 2% horse serum for different period of time. At each time point, cells were incubated with MTT (50 µg/ml) and the formazan crystals were dissolved by adding 100% DMSO. Optical density was taken at 570 nm and normalized with 0 h time point. Bar diagram was plotted as optical density (570 nm) versus time of differentiation (h).

From the MTT assay data, we find that the optical density at 570 nm (which is proportional to number of surviving cells) increases in case of C2C12 cells, whereas in R120-C2C12 cells, the optical density remains more or less constant through out the differentiation time points (Figure 4.3).

4.3.2 Effect of R120G-αB-crystallin mutant on cell cycle regulators and myogenin during differentiation

Terminal cell-cycle arrest is critical for the myogenic transcription and completion of the differentiation program. To decipher the mechanism of inhibition of myogenesis, we compared the cell-cycle profiles of normal C2C12 and R120G-C2C12 myoblasts. We set out to investigate the cell-cycle regulators cyclin D1 and p21. Cyclin D1 and p21 function in an antagonistic manner, cyclin D1 induces G1/S transition, whereas p21 inhibits CDKs and is involved in the cell-cycle arrest (Hunter and Pines, 1994; Walsh and Perlman, 1997). We studied the expression of cyclin D1 and p21 levels at different time points after induction of differentiation of C2C12 cells and R120G-C2C12 cells. Our western blot analysis of lysates of C2C12 cells showed a significant level of cyclin D1 initially upon differentiation (0 h), which decreases sharply to 0.12 fold at 24 h of differentiation, after which the basal level of cyclin D1 was maintained. On the other hand, the level of p21 increased gradually to 8.3 fold at 96 h of differentiation (Figure 4.4A). Interestingly, R120G-C2C12 cells also show a similar trend of decrease in the level of cyclin D1 with concomitant increase in the level of p21 with increasing time of differentiation (Figure 4.4B). Our data, thus, suggests that R120G-C2C12 cells show apparently similar cell-cycle behavior as that of C2C12 cells during differentiation. Further, we performed FACS analysis, and found that ~ 60% of C2C12 cells undergo G0/G1 arrest at 12 h of differentiation, whereas in R120G-C2C12 cells, ~ 83% cells were arrested at the similar time point (data not shown). Importantly, with increasing time of differentiation, unlike normal C2C12 cells, severe cell death was observed in R120G-C2C12.

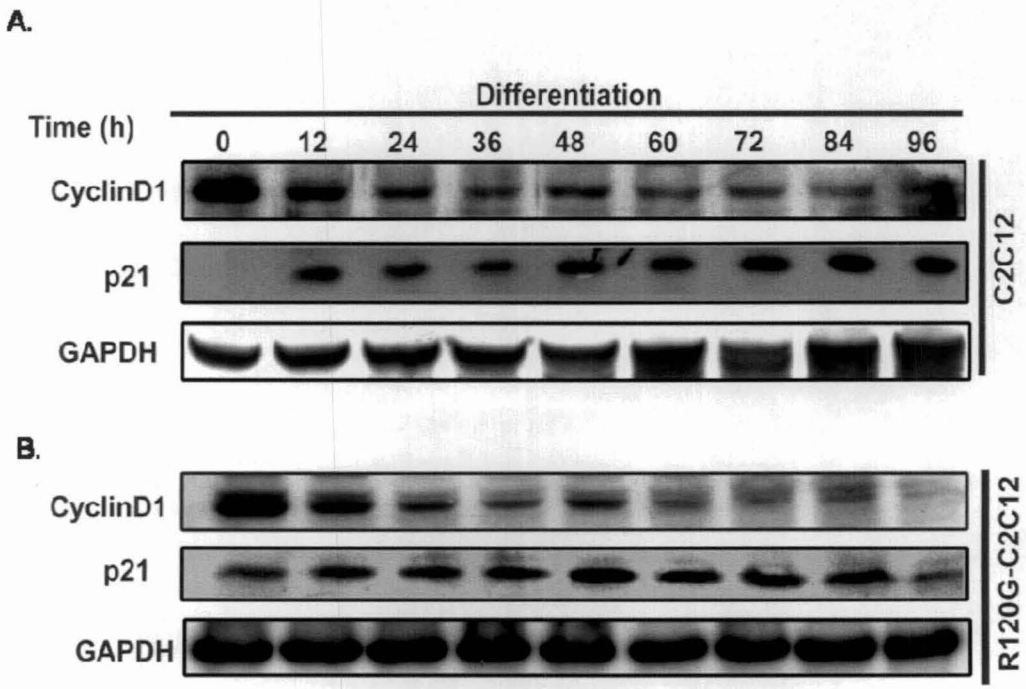


Figure 4.4. Western blots showing cyclin D1 and p21 profile during differentiation of C2C12 and R120G-C2C12 cells. C2C12 and R120G-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** shows the western blot for cyclin D1 and p21 profile from C2C12 cell lysate, **Panel B** shows the western blot for cyclin D1 and p21 from the cells over-expressing R120G-αB-crystallin mutant (R120G-C2C12) lysates. Western for GAPDH, used as loading control is also shown.

To further analyze the inhibition in the differentiation process, we checked differentiation markers such as myogenin. Successful progression through the skeletal muscle cell differentiation program is marked by activation of myogenic regulatory factors (MRFs), including MyoD, Myf5, and myogenin, which control expression of muscle specific genes. The results reported in the previous sections prompted us to investigate whether the inhibition of myogenesis is due to alteration of the expression of muscle regulatory genes. Our immunostaining data for myogenin showed that in C2C12 cells, the myogenin stained cells gradually increased with the time of differentiation (Figure 4.5). On the other hand, the number of cells positive for myogenin in R120G-C2C12 cells continuously decreased with concomitant decrease in total cell population (Figure 4.5). The decrease in the number of myogenin positive population may be attributed to increased cell death observed in R120G-C2C12 cells.

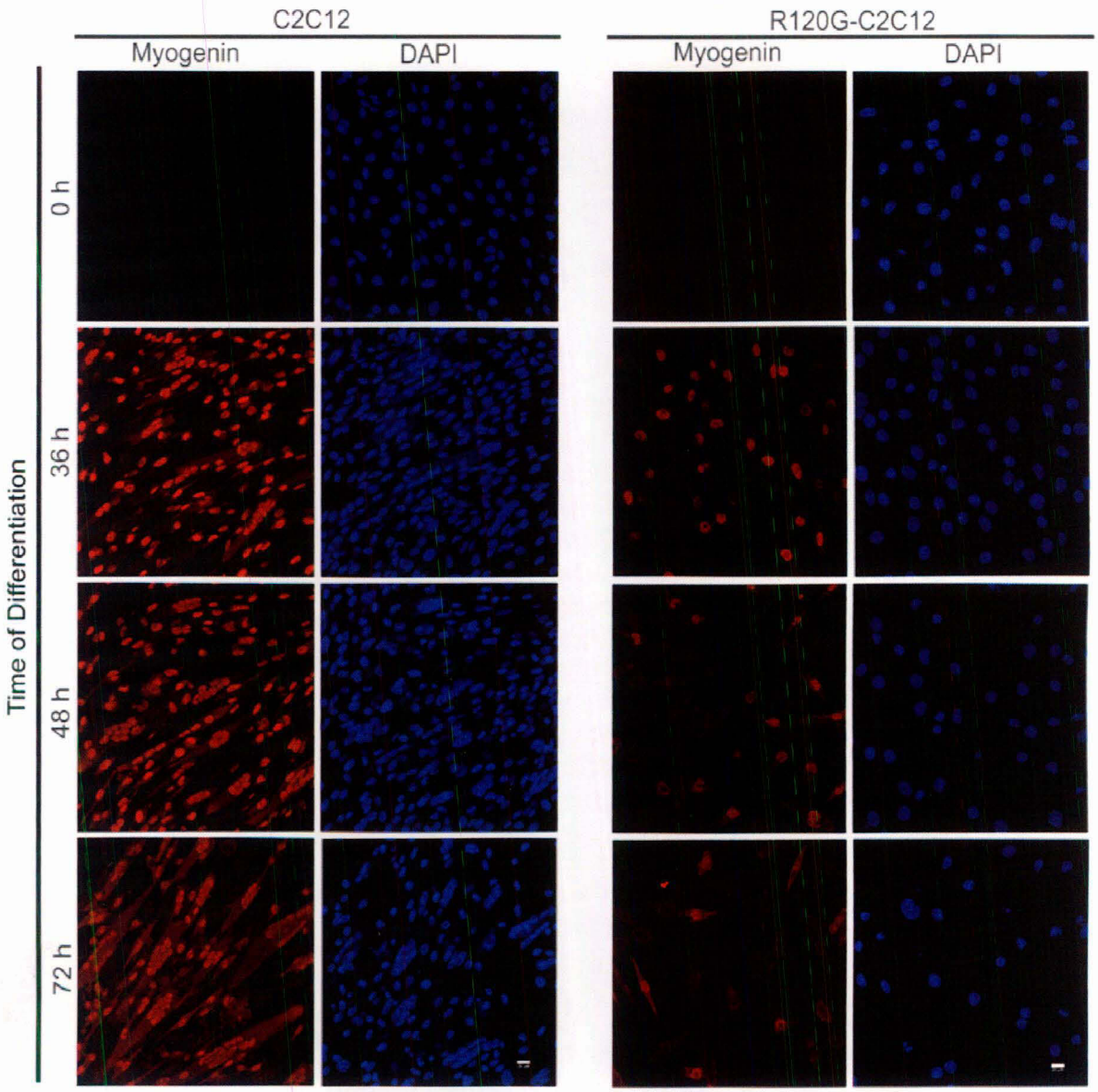


Figure 4.5. Immunostaining of myogenic marker, myogenin in C2C12 and R120G-C2C12 cells during differentiation. C2C12 and R120G-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. Cells were fixed in 3.7% formaldehyde and immunostaining was done using antibodies specific for myogenin in C2C12 and R120G-C2C12 cells at different periods of differentiation.

4.3.3 R120G-αB-crystallin mutant modulates apparent stability of MyoD during muscle differentiation

The results obtained so far demonstrate that over-expression of R120G-αB-crystallin mutant in C2C12 cells (R120G-C2C12) lead to inhibition of myogenesis, although the expression of myogenin and p21 was similar to that in normal C2C12 cells. Various studies show that the R120G-αB-crystallin mutant behaves as a dominant negative mutant as this mutation alters the structure and reduces the chaperone activity of wild-type αB-crystallin (Kumar *et al.,* 1999; Perng *et al.,* 1999; Bova *et al.,* 1999). In the previous chapter (chapter III), we have shown that αB-crystallin enhances the degradation of MyoD and reduces its apparent half-life during the differentiation process (Figure 3.9; chapter III). In order to investigate the effect of R120G-αB-crystallin mutant on the MyoD expression, normal C2C12 and R120G-C2C12 myoblasts were allowed to differentiate and MyoD expression profile was analysed by western blot of total cell lysates. Our western blot analysis shows that in normal C2C12 myoblasts, the level of MyoD increases till 24 h of differentiation and decreases thereafter. R120G-C2C12 cells also showed a gradual increase in MyoD expression till 24 h of differentiation. Interestingly, the decrease in the level of MyoD in R120G-C2C12 was not as fast as that of C2C12 cells at later time points. It is important to note that a significant level of MyoD was observed till 48 h of differentiation in R120G-C2C12 cells as compared to C2C12 cells. (Figure 4.6A and B).

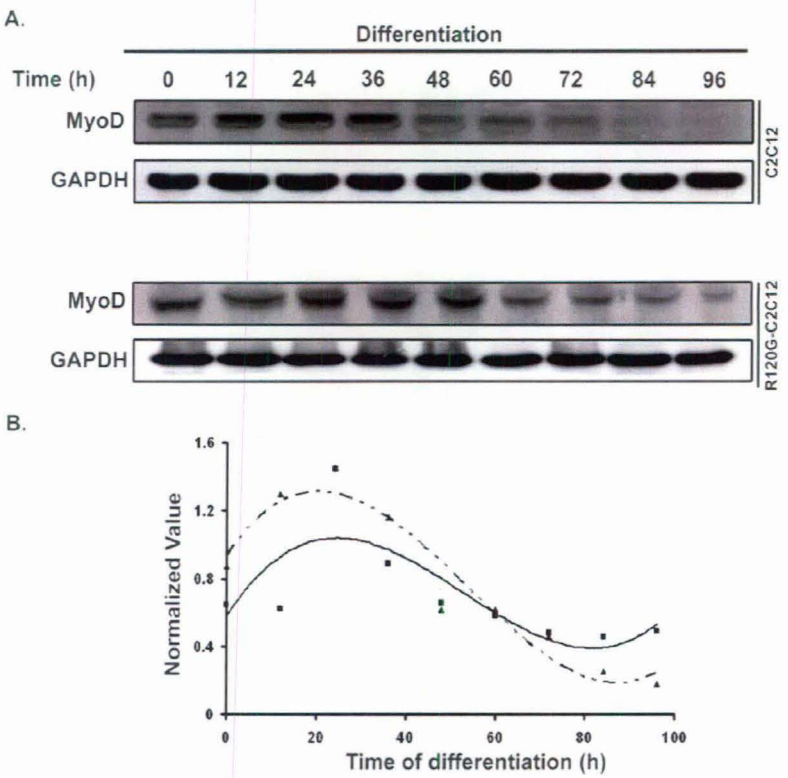


Figure 4.6. MyoD profile in C2C12 and R120G-C2C12 cells during differentiation. C2C12 and R120G-C2C12 cells were induced to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** shows the western blot for MyoD expression profile from C2C12 and cells overexpressing R120G-αB-crystallin mutant (R120G-C2C12) lysates. Western blot for GAPDH was used as loading control. **Panel B** shows the graphic representation of normalized band intensities from C2C12 (▲) and R120G-C2C12 (■).

To gain further insight into the sustained level of MyoD in R120G-C2C12 cells, we carried out experiments to determine the apparent half-life of MyoD in R120G-C2C12 cells during the differentiation process. MyoD is a short-lived protein and gets degraded by the 26S proteasome pathway (Abu Hatoum *et al.,* 1998; Breitschopf *et al.,* 1998); we compared the apparent half-life of endogenous MyoD protein in both C2C12 and R120G-C2C12 cells. Normal C2C12 and R120G-C2C12 myoblasts were incubated in differentiating medium (DM) for 24 h and then treated either with 50 μg/ml of cycloheximide, (CHX, a protein synthesis inhibitor) alone or in combination with MG132, a 26S proteasomal inhibitor (10

μM). As shown in figure 4.7, MyoD gets degraded in C2C12 cells with an apparent half-life of 2.37 h ($t_{1/2} \approx 2.37$ h). Interestingly, in R120G-C2C12 cells, the degradation of MyoD was significantly reduced with apparent half-life being 3.01 h ($t_{1/2} \approx 3.01$ h) (Figure 4.7B and D).

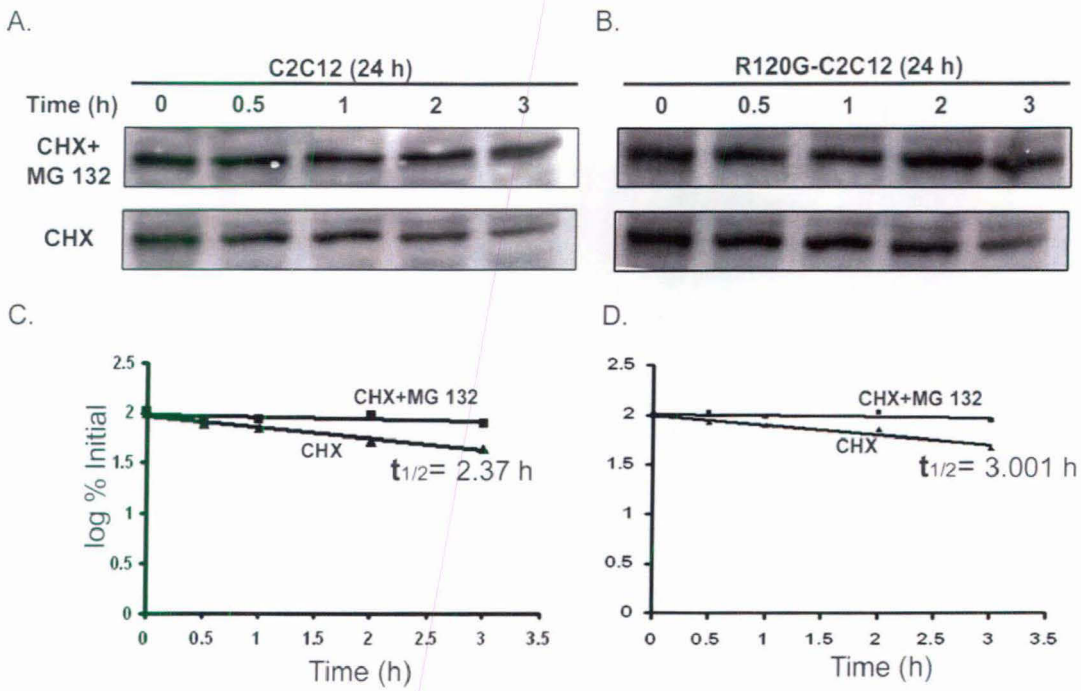


Figure 4.7. Apparent Half-life of endogenous MyoD in C2C12 and R120G-C2C12 cells during differentiation. C2C12 and R120G-C2C12 cells were induced to differentiate till 24 h of time. Further, cells were treated either with CHX alone or in combination with MG132. Cells were harvested, lysed at 0, 0.5, 1, 2, and 3 h and were evaluated via SDS-PAGE and western blot for MyoD protein. **Panel A** and **B** shows the western blot of MyoD from the cell lysates of C2C12 and R120G-C2C12 cells respectively. The pixels for each band were measured and normalized so that the number of pixels at $t = 0$ was 100%. The $\log_{10}$ of the percentage of pixels was plotted *versus* time, and the $t_{1/2}$ was calculated from the $\log_{10}$ of 50%. **Panel C** and **D** represent the apparent half-life of MyoD during the differentiation of C2C12 and R120G-C2C12 cells respectively.

4.3.4 Expression profile of αB-crystallin in R120G-C2C12 cells during differentiation

In order to study the effect on the expression of αB-crystallin in R120G-C2C12 cells during the differentiation process, we allowed C2C12 and R120G-C2C12 cells to

differentiate for different time points and the cell lysates were probed with antibodies specific for αB-crystallin. Our western blot analysis shows that while the level of αB-crystallin increases upon differentiation in C2C12 cells (Figure 4.8A), it remained almost unchanged in R120G-C2C12 cells at all the time points studied (Figure 4.8B).

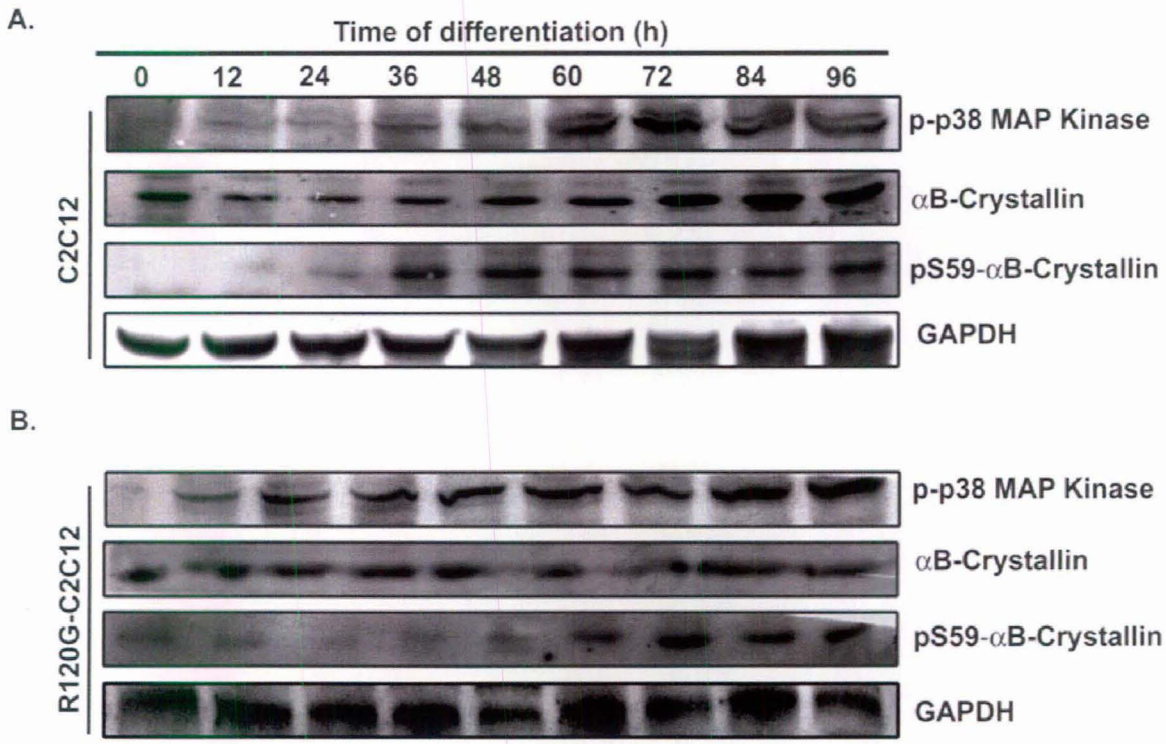


Figure 4.8. Western blots showing p-p38-MAPK, αB-crystallin and phospho-Ser-59-αB-crystallin profile during differentiation of C2C12 and R120G-C2C12 cells. C2C12 and R120G-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. **Panel A** shows the profile of phospho-p38 MAPK, αB-crystallin and phospho-Ser-59-αB-crystallin from C2C12 lysates and **Panel B** shows the western blot of phospho-p38 MAPK, αB-crystallin and phospho-Ser-59-αB-crystallin profile from R120G-C2C12 cell lysates.

Further we investigated the phosphorylation status of αB-crystallin using antibodies specific for phospho-Ser-59-αB-crystallin and phospho-Ser-45-αB-crystallin at different time points of differentiation in both cell types. We found that there was a gradual increase in the phospho-Ser-59-αB-crystallin with no phosphorylation at Ser-45 residue (Figure 4.8). Importantly, the phospho-Ser-59-αB-crystallin was detected at earlier time point in C2C12 cells compared to R120G-C2C12 cells (Figure 4.8A and B).

Since p38-MAPK is known to phosphorylate Ser-59 residue of αB-crystallin (Ito *et al.*, 1997), we have investigated the phosphorylated p38-MAP kinase profile during differentiation. Our western blot analysis for phospho-p38 MAPK showed that the phospho-

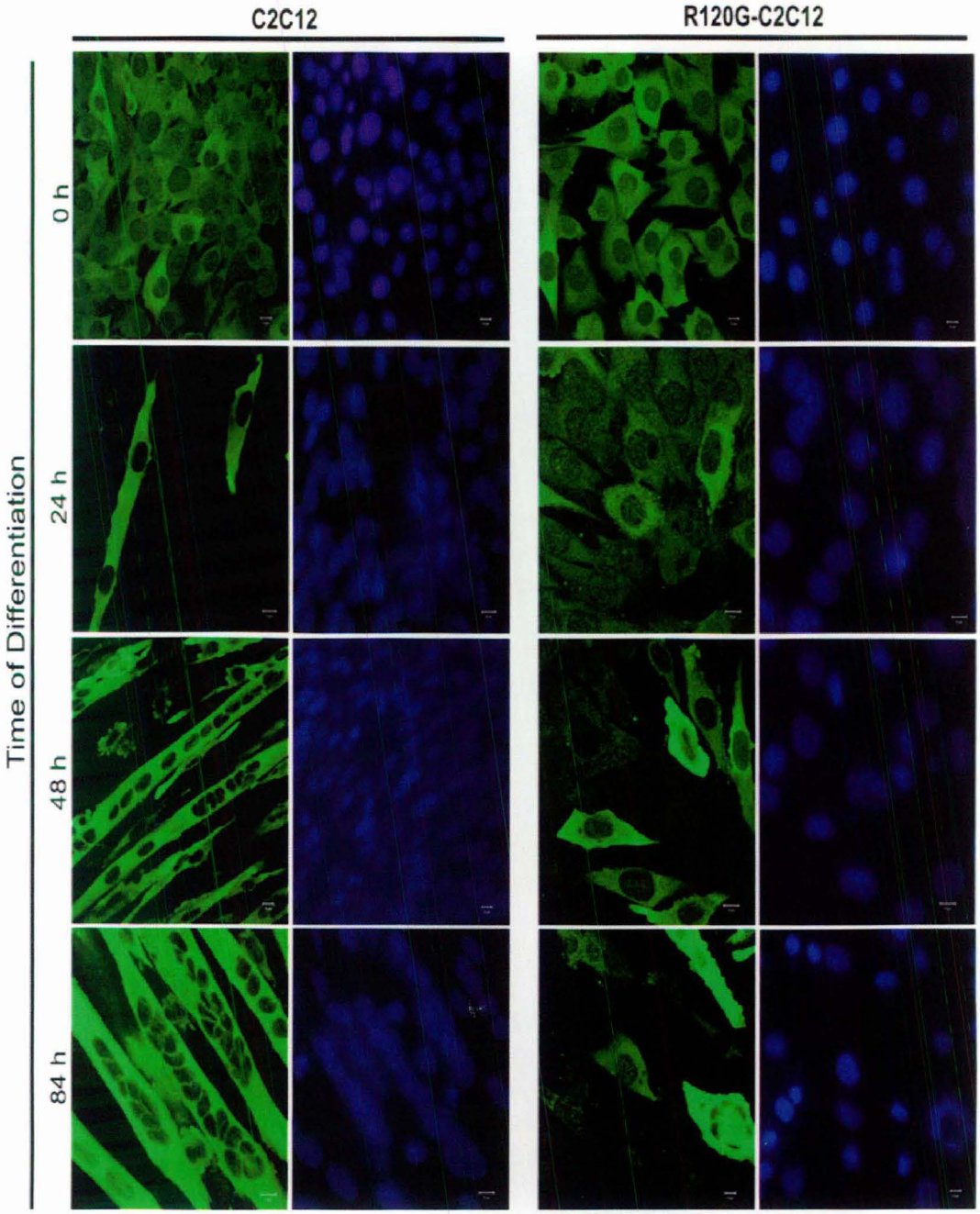


Figure 4.9. Immunolocalization of αB-crystallin during differentiation. C2C12 and R120G-C2C12 myoblasts, grown on coverslips were induced to differentiate for different period of time. The cells were fixed in 3.7% (v/v) formaldehyde and immunostained using αB-crystallin-specific antibodies. The nuclei were counterstained with DAPI. The scale bar represents 10 μm.

p38-MAPK level increases with increasing time of differentiation in both C2C12 cells as well as in R120G-C2C12 cells (Figure 4.8).

The R120G-αB-crystallin mutant is known to form aggregates both *in vitro* as well as *in vivo* (Perng *et al.,* 1999; Wang *et al.,* 2001). In order to visualize the effect of the R120G mutant, we analyzed the localization of αB-crystallin upon differentiation. Our immunostaining data shows that initially upon differentiation (0 h), αB-crystallin is localized predominantly in the cytoplasm with some speckle-like staining in the nucleus of both C2C12 and R120G-C2C12 myoblasts. Interestingly, with increasing time of differentiation, αB-crystallin localizes exclusively to the cytoplasm with no speckle-like staining in the nucleus of C2C12 as well as R120G-C2C12 cells (Figure 4.9). In addition, we find that cells with R120G mutants form a sac-like structure at the later stages of differentiation (84 h) (Figure 4.9). The formation of sac-like structure could be due to its inability to modulate cytoskeletal dynamics and/or desmin assembly.

4.3.5 R120G-αB-crystallin mutant leads to enhanced caspase-3 activation during muscle differentiation

During skeletal muscle differentiation, a subset of myogenic precursor cells acquires apoptotic resistance and is destined to undergo terminal differentiation. The myopathy-causing αB-crystallin mutant is shown to induce apoptotic pathway in cardiac muscle and altered contractile mechanics (Sanbe *et al.,* 2004). Kamradt *et al.,* (2002) have shown that the R120G-αB-crystallin mutant is defective in preventing proteolytic cleavage of pro-caspase-3. Caspase-3 activation is required for skeletal muscle differentiation but an over-active caspase-3 may be detrimental to the cells. We monitored the caspase-3 cleavage in C2C12 and R120G-C2C12 cells. Our western blot analysis suggests that caspase-3 gets activated and cleaved to p17/p12 (active caspase-3) in the normal C2C12 cells (Figure 4.10). This activation of caspase-3 may be necessary for the proper differentiation process to occur. Compared to normal C2C12 cells, R120G-C2C12 cells exhibit greater cleavage of pro-caspase-3 into active caspase-3 (p17/12) at similar time points, which accumulate gradually with increasing time of differentiation (Figure 4.10).

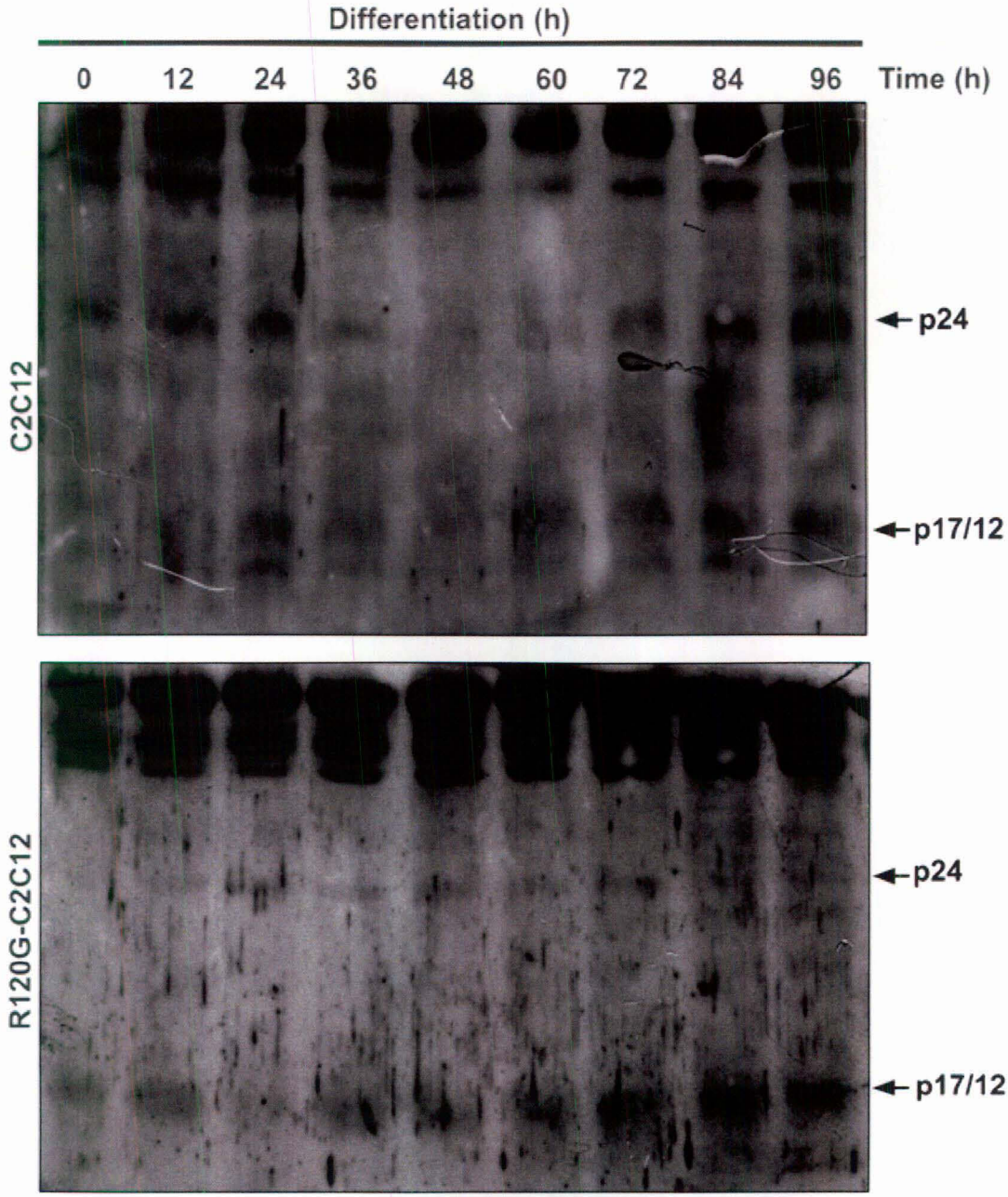


Figure 4.10. Western blots showing caspase-3 activation profile during C2C12 and R120G-C2C12 cells differentiation. C2C12 and R120G-C2C12 cells were allowed to differentiate in DMEM medium containing 2% horse serum and harvested at different time-points of differentiation. Western blot analysis for caspase-3 cleavage was perfomed using C2C12 and R120G-C2C12 cell lysates. The arrows indicate the cleaved products from pro-caspase-3 to form pro-active caspase-3 (p24) and active caspase-3 (p17/12).

4.4 Discussion

Desmin related myopathy (DRM) is a neuromuscular disorder characterized by large intracellular protein aggregates leading to cell death (Perng *et al.,* 2004; Maloyan *et al.,* 2005). Aggregates of intermediate filament together with αB-crystallin have been reported in biopsies of muscle tissue from DRM patients (Nédellec *et al.,* 2002). Importantly, other diseases, such as Alexander's disease and drug-induced hepatitis, are also characterized by intermediate filament aggregates (Reichard *et al.,* 1996; Magin *et al.,* 1998). These aggregates are typically co-associated with αB-crystallin, despite the fact that they involve different intermediate filament proteins (Lowe *et al.,* 1992). This suggests that the association of αB-crystallin with intermediate filament aggregates is a generic response to the pathological rearrangement of intermediate filaments and not dependent on specific intermediate filament proteins. Notably, although many mutations in the intermediate filament protein desmin have been linked with DRM, so far only a single mutation, R120G, in αB-crystallin has been identified (Goldfarb *et al.,* 1998; Munoz-Marmol *et al.,* 1998; Vicart *et al.,* 1998). It is suggested that, in α-crystallinopathy, desmin collapses with R120G-αB-crystallin mutant due to a reduced chaperone activity of mutant protein (Perng *et al.,* 1999; Perng *et al.,* 2004). Moreover, chaperone activity of αB-crystallin seems to be necessary for the proper organization of the desmin filaments (Nicholl and Quinlan, 1994). Interestingly, desmin- and αB-crystallin-null mice as well as mice expressing desmin mutants all have a much less severe myopathy than those expressing R120G-αB-crystallin mutant, suggesting that R120G-αB-crystallin induced myopathy might result from other factors in addition to the loss of function of desmin or αB-crystallin (Wang *et al.,* 2001; Chávez Zobel *et al.,* 2003). Desmin is expressed during the development of skeletal, cardiac and smooth muscle, and endothelial cells (Costa *et al.,* 2004). In avian cells, desmin is the first muscle-specific cytoskeletal protein or isoform to be expressed, after (or together with) the commitment of precursor cells to the muscle lineage (Li and Capetanaki, 1993). The effect of R120G-αB-crystallin mutant on the muscle differentiation process and desmin organization is not known so far.

This chapter describes our investigation on the effect of R120G-αB-crystallin during muscle differentiation. We show that R120G-αB-crystallin mutant inhibits the process of myogenesis in C2C12 mouse myoblasts (Figure 4.1 and Figure 4.2). We found that in

R120G-C2C12 cells the profile of cell cycle regulators such as cyclin D1 and p21 was similar to normal C2C12 cells (Figure 4.4). Further, cell cycle analysis shows that approximately 83% R120G-C2C12 cells were G0/G1-arrested at as early as 12 h of differentiation compared to ~ 60% in C2C12 cells (Figure 4.5). This could be due to the maintenance of a sustained level of MyoD over a longer period of time in R120G-C2C12 cells. It is known that during differentiation, the expression of p21 is MyoD-dependent (Guo *et al.,* 1995; Halevy *et al.,* 1995). Moreover, a recent study from our laboratory has shown that R120G-αB-crystallin mutant negatively regulates NF-κB activity (unpublished data). Therefore, it is possible that R120G-αB-crystallin mutant induced deactivation of NF-κB may lead to reduced cyclin D1 expression, as it has been shown that activated NF-κB leads to increase in cyclin D1 expression (Guttridge *et al.,* 1999). The expression of cyclin D1 and MyoD is highly linked during the differentiation process in an antagonistic manner (Rao *et al.,* 1994). We find that in C2C12 cells, the expression of MyoD increases till 24 h and decreases sharply at 48 h of differentiation. Interestingly, in R120G-C2C12 cells, the increase in expression of MyoD was similar to that in C2C12 cells, reaching a maximum at 24 h of differentiation; however, the decrease was not as fast as that in C2C12 cells (Figure 4.5). Our study suggests that in R120G-C2C12 cells, the apparent half-life of MyoD was relatively higher ($t_{1/2}$= 3.01 h) compared to that in C2C12 cells ($t_{1/2}$= 2.37 h) (Figure 4.6). It is important to note that αB-crystallin has been shown to be a part of the FBX4-Ub complex and promotes FBX4 dependent ubiquitination (den Engelsman *et al.,* 2003). It can be speculated that R120G mutant, being dominant negative, inhibits the ubiquitination process, thereby stabilizing MyoD protein. During the differentiation process, we show that αB-crystallin gets phosphorylated at Ser-59 residue (Figure 4.7). Whether this preferential phosphorylation is playing a role in the MyoD ubiquitination needs to be investigated. Interestingly, the R120G-αB-crystallin mutant is known to get hyperphosphorylated under normal condition (den Engelsman *et al.,* 2005), however, a recent work from our laboratory has shown that upon exposure to TNF-α, the phosphorylation of R120G-αB-crystallin decreases (unpublished data). The phosphorylation at Ser-59 is known to be induced by the activation of p38-MAPK (Ito *et al.,* 1997). As expected the phospho-p38 MAPK gradually increased in both C2C12 and R120G-C2C12 cells (Figure 4.7). Hence our results suggest that the pathway upstream to αB-crystallin phosphorylation is not affected in the cells over-expressing the R120G-αB-crystallin mutant. The localization studies of αB-crystallin in C2C12 cells and R120G-C2C12 cells, show similar staining of αB-crystallin in the cytoplasm (Figure 4.8). Although the

R120G-C2C12 cells form sac-like structures, αB-crystallin remains exclusively in the cytoplasm. Unlike myoblasts, no speckle-like staining in the nucleus was observed at the later stages of differentiation (Figure 4.8). It is important to note that during the differentiation of R120G-C2C12 cells, we found an extensive cell death. We have shown that there is a greater accumulation of active caspase-3 (p17/12) in R120G-C2C12 cells than that in normal C2C12 cells (Figure 4.9). Mao *et al.,* have shown that R120G-αB-crystallin has reduced ability to bind to Bax and Bcl-X(s), thereby supporting the point that the mutant has reduced protective ability compared to the wild-type αB-crystallin. Taken together, we find that the R120G-αB-crystallin mutant has a large detrimental effect during the differentiation process, which may be responsible for the observed myopathic condition. Strategies, which can reduce specifically the mutant expression, may be useful in the treatment of patients with R120G-αB-crystallin mutant.

CHAPTER 5

Concluding Remarks

5.1 Concluding Remarks

Small heat shock proteins (sHSPs) are a ubiquitous class of molecular chaperones that are present from lower prokaryotes to higher eukaryotes such as mammals. The expression of these sHSPs gets enhanced under stress as well as in disease condition.

Among all the sHSPs known, HSP27 and α-crystallin are by far the most studied sHSPs. Work from our laboratory over the years has resulted in considerable insight into the structure-function aspects of α-crystallin. α-Crystallin has been shown to act as a molecular chaperone and confer stress tolerance by interacting with aggregation-prone intermediates. In addition to its chaperone-like activity, αA- and αB-crystallins appears to have role in many cellular events such as cell division, motility, apoptosis and differentiation. Recent reports from knockout and mutant studies suggest critical involvement of αB-crystallin in the maintenance of muscle homeostasis and regulation of cytoskeletal dynamics.

αB-crystallin appears to have a role in modulating the dynamics of contractile proteins, and is an essential component of the cardiac muscle and skeletal muscle. This may explain its relatively high expression level (~ 3 % of total cell protein) in muscle tissues. Disruption of actin microfilament is an early effect of stresses such as heat and altered pH conditions.We have investigated the role of αB-crystallin in regulating actin filament dynamics using H9C2, a rat cardiomyoblasts cell line and neonatal rat ventricular myocytes (NRVMs). Our results show increased expression of αB-crystallin in these cells during stress conditions. We have carried out experiments to decipher the role of endogenous αB-crystallin in modulating actin stability during heat stress. Our results show that αB-crystallin exhibits fiber-like appearance in the cytoplasm and associates with actin filaments upon heat stress. The association of αB-crystallin with F-actin is dependent on its phosphorylation at Ser-45 and Ser-59 residues. Phosphorylation seems to be an essential event for the association of αB-crystallin, as inhibition of phosphorylation abrogates its ability to interact with actin filaments. Further, we find that the association is functionally relevant since heat pretreatment of cells lead to failure of cytochalasin B-induced depolymerization of actin. We also provide evidence

that αB-crystallin maintains the functional integrity in processes like pinocytosis, of the actin microfilament during heat stress. Our study provides interesting insights into the functionality of αB-crystallin in maintenance of cellular architecture by modulating actin filament dynamics *in vivo* under stress conditions.

Modulation or reorganization of cytoskeletal proteins has also been documented during the differentiation process. In addition, muscle differentiation process is also associated with significant increase in the expression of sHSPs such as αB-crystallin and HSP27. It is important to note that muscle constitutively expresses as many as six sHSPs. Whether, all of them work in a synergistic fashion or in an independent manner is yet to be deciphered. The role of αB-crystallin appears to be significant as mice lacking αB-crystallin have lower muscle mass. These observations raise interesting questions; Does αB-crystallin have any role to play in muscle development and maintenance of muscle homeostasis? Since αB-crystallin is shown to have anti-apoptotic function during myogenesis, is it possible to cure diseases like cachexia by its over-expression?

We have investigated the role of αB-crystallin during the muscle differentiation process using C2C12, a mouse myoblasts cell line. Surprisingly, we find that over-expression of αB-crystallin leads to delay in myogenesis. Our studies show that αB-crystallin alters the cell-cycle arrest and continues proliferation by modulating the cell cycle regulators such as cyclin D1 and p21. The expression of cyclin D1 is enhanced and sustained for a longer period of time while the p21 expression was delayed and reduced in CRYAB-C2C12 cells compared to those of C2C12 cells. Further, we find that MyoD dependent activation of p21 and myogenin was delayed more than 48 h of differentiation. We show that αB-crystallin shifts the expression maximum of MyoD, the master regulator of muscle differentiation by 12 h during the differentiation process. Interestingly, we find that αB-crystallin leads to enhanced ubiquitination, leading to faster degradation of MyoD (reduced apparent half-life). We believe that the shift in the MyoD maximum is due to the combined effect of reduced synthesis and enhanced degradation in CRYAB-C2C12 cells. Our results show that αB-crystallin gets phosphorylated specifically at the Ser-59 residue during muscle differentiation. It is not known, whether phosphorylation of αB-crystallin has any role to play during this process. Taken together,

our results suggest that αB-crystallin is essential for muscle differentiation and regulation of αB-crystallin is very important for proper myogenesis.

In addition, we have carried out experiments to investigate the effect of a point mutation in αB-crystallin (R120G) in muscle differentiation. The mutant αB-crystallin (R120G-αB-crystallin) is a dominant negative mutant and is shown to be associated with desmin related myopathy. Studies with R120G-αB-crystallin suggest that the expression profile of p21, myogenin and MyoD were similar to normal C2C12 cells, indicating that R120G-αB-crystallin mutant does not have any effect in the initiation of differentiation program. Interestingly, we find that the mutant leads to an extensive cell death during the differentiation process. The aggregation-induced apoptosis could be one possible reason for the increased cell death. However, the mechanistic details of enhanced cell death in R120G-C2C12 cells remain to be investigated.

5.2 Possible future directions:

αB-crystallin, a molecular chaperone, is found in significant amount in brain, kidney and muscle tissue. In muscle it comprises up to 3 % of the total cell protein. The review of literature indicates that αB-crystallin has many more functions to perform than previously thought.

In an attempt to decipher the *in vivo* function of αB-crystallin, we chose muscle cells as model system. Our study using H9C2 myoblasts and NRVMs demonstrate that αB-crystallin protects actin microfilaments during heat stress in a phosphorylation dependent manner. Whether knocking out or reducing the level of αB-crystallin using RNAi approach would result in the collapse of the actin microfilaments needs to be investigated. It is possible that the ortholog of αB-crystallin, HSP25 also exhibit similar behavior in heat-stressed condition; therefore it is likely that both αB-crystallin and HSP25 may act in concert. Further, upon reducing the level of αB-crystallin, HSP25 may take over its function. Hence it may be necessary to silence HSP25 as well, to investigate the effect of absence of αB-crystallin on the microfilament network. A point mutant, R120G-αB-crystallin is known to have altered structure and reduced chaperone

activity. It has been found with aggregates of desmin and is attributed in desmin related myopathy. It would be interesting to investigate the ability of the mutant αB-crystallin in providing stability to the actin filaments.

The role of αB-crystallin in the differentiation process is far from clear. We have tried to explore the possible function/s of αB-crystallin during the muscle differentiation process. Surprisingly, we find that αB-crystallin delays the differentiation process to a significant extent. We demonstrated that αB-crystallin alters the temporal profile of cell cycle regulators and stability of MyoD. It is not clear, whether reducing the αB-crystallin expression would results in enhancing the differentiation process. Importantly, it would be interesting to know what is the effect of αB-crystallin on the deactivators of MyoD such as Id1 and/or other E-box proteins?

Investigating into the effect of R120G, a myopathy causing mutant of αB-crystallin, we find that it leads to severe cell death. Although the initiation of differentiation programme was similar to C2C12 cells, these R120G-C2C12 cells were unable to form myotubes due to extensive cell death. We have explored the possible pathways of inducing cell death in αB-R120G mutant background. Our preliminary results suggest that R120G-αB-crystallin mutant leads to ER-stress. This observation is further supported by the enhanced expression of ER chaperones Grp94, in cells over-expressing R120G-αB-crystallin during the differentiation process. ER stress-induced cell death might be due to or independent of aggregation-induced cytotoxicity. We find that treatment of cells with Brefeldin A, an ER stress inducer, causes severe cell death. Moreover, the ER-stress induced cell death was significantly reduced in cells over-expressing αB-crystallin, indicating that αB-crystallin may rescue cells from ER stress. Brefeldin A treatment leads to increase in the cytosolic Ca^{2+} in C2C12 cells, we find that this increase in cytosolic Ca^{2+} is higher in the cells expressing R120G-αB-crystallin compared to C2C12 cells or C2C12 cells expressing wild type αB-crystallin. The role of ER stress and ER stress-related processes in αB-crystallin mediated functions needs to be investigated.

Bibliography

Abravaya K, Myers MP, Murphy SP, and Morimoto RI. (1992) The human heat shock protein hsp70 interacts with HSF, the transcription factor that regulates heat shock gene expression. Genes and Dev. **6:** 1153–1164.

Abu Hatoum O, Gross-Mesilaty S, Breitschopf K, Hoffman A, Gonen H, Ciechanover A, and Bengal E. (1998) Degradation of myogenic transcription factor MyoD by the ubiquitin pathway in vivo and in vitro: regulation by specific DNA binding. Mol Cell Biol. **18:** 5670-5677.

Adhikari AS, Rao KS, Rangaraj N, Parnaik VK, and Rao CM. (2004) Heat stress-induced localization of small heat shock proteins in mouse myoblasts: intranuclear lamin A/C speckles as target for alphaB-crystallin and Hsp25. Exp Cell Res. **299:** 393-403.

Ahmad MF, Raman B, Ramakrishna T, and Rao CM. (2008) Effect of phosphorylation on alpha B-crystallin: differences in stability, subunit exchange and chaperone activity of homo and mixed oligomers of alpha B-crystallin and its phosphorylation-mimicking mutant. J Mol Biol. **375:** 1040-51.

Ali A, Bharadwaj S, O'Carroll R, and Ovsenek N. (1998). HSP90 interacts with and regulates the activity of heat shock factor 1 in *Xenopus* oocytes. Mol Cell Biol. **18:** 4949– 4960.

Allen SP, Polazzi JO, Gierse JK, and Easton AM. (1992) Two novel heat shock genes encoding proteins produced in response to heterologous protein expression in Escherichia coli. J Bacteriol. **174:** 6938-47.

Amack JD, and Mahadevan MS. (2004) Myogenic defects in myotonic dystrophy. Dev Biol. **265:** 294-301.

Andley UP, Song Z, Wawrousek EF, Fleming TP, and Bassnett S. (2000) Differential protective activity of alpha A- and alphaB-crystallin in lens epithelial cells. J Biol Chem. **275:** 36823–36831.

Andrés V, and Walsh K. (1996) Myogenin expression, cell cycle withdrawal, and phenotypic differentiation are temporally separable events that precede cell fusion upon myogenesis. J Cell Biol. **132:** 657-66.

Anfinsen CB, and Haber E. (1961) Studies on the Reduction and Re-formation of Protein Disulfide Bonds. J Biol Chem. **236:** 1361–1363.

Anfinsen CB. (1973) Principles that govern the folding of protein chains. Science. **181:** 223-230.

Anker SD, Ponikowski P, Varney S, Chua TP, Clark AL, Webb-Peploe KM, Harrington D, Kox WJ, Poole-Wilson PA, and Coats AJ. (1997) Wasting as independent risk factor for mortality in chronic heart failure. Lancet. **349:** 1050-3.

Arrigo AP. (2000) sHsp as novel regulators of programmed cell death and tumorigenicity. Pathol Biol. **48:** 280–288.

Baeuerle PA. (1995) Inducible gene expression. Vol. I. Environmental stresses and nutrients.

Bai F, Xi J, Higashikubo R, and Andley UP. (2004a) A comparative analysis of alphaA- and alphaB-crystallin expression during the cell cycle in primary mouse lens epithelial cultures. Exp Eye Res. **79:** 795–805.

Bai F, Xi J, Higashikubo R, and Andley UP. (2004b) Cell kinetic status of mouse lens epithelial cells lacking alphaA- and alphaB-crystallin. Mol Cell Biochem. **265:** 115-22.

Bai F, Xi JH, Wawrousek EF, Fleming TP, Andley UP. (2003) Hyperproliferation and p53 status of lens epithelial cells derived from alphaB-crystallin knockout mice. J Biol Chem. **278:** 36876-86.

Baler R, Welch WJ, and Voellmy R. (1992). Heat shock gene regulation by nascent polypeptides and denatured proteins: hsp70 as a potential autoregulatory factor. J Cell Biol. **117:** 1151–1159.

Bardwell JC, and Craig EA. (1987) Eukaryotic Mr 83,000 heat shock protein has a homologue in *Escherichia coli*. Proc Natl Acad Sci. **84:** 5177–5181.

Bardwell JC, and Craig EA. (1988) Ancient heat shock gene is dispensable. J Bacteriol. **170:** 2977–2983.

Bar-Sagi D, and Feramisco JR. (1986) Induction of membrane ruffling and fluid-phase pinocytosis in quiescent fibroblasts by ras proteins. Science. **233:** 1061–1068.

Beall A, Bagwell D, Woodrum D, Stoming TA, Kato K, Suzuki A, Rasmussen H, and Brophy CM. (1999) The small heat shock-related protein, HSP20, is phosphorylated on serine 16 during cyclic nucleotide-dependent relaxation. J Biol Chem. **274:** 11344–11351.

Bellyei S, Szigeti A, Boronkai A, Pozsgai E, Gomori E, Melegh B, Janaky T, Bognar Z, Hocsak E, Sumegi B, and Gallyas F Jr. (2007a) Inhibition of cell death by a novel 16.2 kD heat shock protein predominantly via Hsp90 mediated lipid rafts stabilization and Akt activation pathway. Apoptosis. **12:** 97–112.

Bellyei S, Szigeti A, Pozsgai E, Boronkai A, Gomori E, Hocsak E, Farkas R, Sumegi B, and Gallyas F Jr. (2007b) Preventing apoptotic cell death by a novel small heat shock protein. Eur J Cell Biol. **86:** 161-171.

Bennardini F, Wrzosek A, and Chiesi M. (1992) Alpha B-crystallin in cardiac tissue. Association with actin and desmin filaments. Circ Res. **71:** 288-294.

Benndorf R, Hayess K, Ryazantsev S, Wieske M, Behlke J, and Lutsch G. (1994) Phosphorylation and supramolecular organization of murine small heat shock protein HSP25 abolish its actin polymerization-inhibiting activity. J. Biol. Chem. **269:** 20780–20784.

Bhat SP, and Nagineni CN. (1989) alpha B subunit of lens-specific protein alpha-crystallin is present in other ocular and non-ocular tissues. Biochem Biophys Res Commun. **158:** 319-25.

Bhattacharyya T, Karnezis AN, Murphy SP, Hoang T, Freeman BC, Phillips B, and Morimoto RI. (1995) Cloning and subcellular localization of human mitochondrial hsp70. J Biol Chem. **270:** 1705-10.

Birkhauser, and Boston, MA. (1995) Inducible gene expression.Vol. II. Hormonal signals.

Bloemendal H, (1981) Molecular and cellular biology of the eye lens. John Wiley & Sons, New York.

Bloemendal H. (1982) Lens proteins. CRC Crit Rev Biochem. **12:** 1-38.

Bloemendal H. (1977) The vertebrate eye lens. Science. **197:** 127-138.

Boatright KM, and Salvesen GS. (2003) Mechanisms of caspase activation. Curr Opin Cell Biol. **15:** 725–731.

Boelens WC, Van Boekel MA, and de Jong WW. (1998) HspB3, the most deviating of the six known human small heat shock proteins. Biochim Biophys Acta. **1388:** 513-516.

Borkman RF, Knight G, and Obi B. (1996) The molecular chaperone a-crystallin inhibits UV-induced protein aggregation. Exp Eye Res. **62:** 141-148.

Boston RS, Viitanen PV, and Vierling E. (1996) Plant Molecular chaperones and protein folding in plants. Mol Biol. **32:** 191-222.

Bova MP, Huang Q, Ding L, and Horwitz J. (2002) Subunit exchange, conformational stability, and chaperone-like function of the small heat shock protein 16.5 from Methanococcus jannaschii. J Biol Chem. **277:** 38468-38475.

Bova MP, Yaron O, Huang Q, Ding L, Haley DA, Stewart PL, and Horwitz J. (1999) Mutation R120G in alphaB-crystallin, which is linked to a desmin related myopathy, results in an irregular structure and defective chaperone-like function. Proc Natl Acad Sci. **96:** 6137–6142.

Brady JP, Garland D, Duglas-Tabor Y, Robison WG Jr, Groome A, and Wawrousek EF. (1997) Targeted disruption of the mouse alpha A-crystallin gene induces cataract and cytoplasmic inclusion bodies containing the small heat shock protein alpha B-crystallin. Proc Natl Acad Sci. **94:** 884-889.

Brady JP, Garland DL, Green DE, Tamm ER, Giblin FJ, and Wawrousek EF. (2001) AlphaB-crystallin in lens development and muscle integrity: a gene knockout approach. Invest. Ophthalmol Vis Sci. **42:** 2924-2934.

Braun T, and Arnold HH. (1996) Myf-5 and myoD genes are activated in distinct mesenchymal stem cells and determine different skeletal muscle cell lineages. EMBO J. **15:** 310-318.

Breitschopf K, Bengal E, Ziv T, Admon A, and Ciechanover A. (1998) A novel site for ubiquitination: the N-terminal residue, and not internal lysines of MyoD, is essential for conjugation and degradation of the protein. EMBO J. **17:** 5964-5973.

Brown SA, and Kingston RE. (1997) Disruption of downstream chromatin directed by a transcriptional activator. Genes & Dev. **11:** 3116–3121.

Brown Z, Ponce A, Lampi K, Hancock L, and Takemoto L. (2007) Differential binding of mutant (R116C) and wildtype alphaA crystallin to actin. Curr Eye Res. **32:** 1051-1054.

Bryantsev AL, Loktionova SA, Ilyinskaya OP, Tararak EM, Kampinga HH, and Kabakov AE. (2002) Distribution, phosphorylation, and activities of Hsp25 in heat-stressed H9c2 myoblasts: a functional link to cytoprotection. Cell Stress Chaperones. **7:** 146-155.

Bryngelson JD, Onuchic JN, Socci ND, and Wolynes PG. (1995) Funnels, pathways, and the energy landscape of protein folding: a synthesis. Proteins. **21:** 167-95.

Buck M, and Chojkier M. (1996) Muscle wasting and dedifferentiation induced by oxidative stress in a murine model of cachexia is prevented by inhibitors of nitric oxide synthesis and antioxidants. EMBO J. **15:** 1753–1765.

Bukau B, and Horwich AL. (1998) The Hsp70 and Hsp60 chaperone machines. Cell. **92:** 351-366.

Capetanaki Y, Milner DJ, and Weitzer G. (1997) Desmin in muscle formation and maintenance: knockouts and consequences. Cell Struct Funct. **22:** 103-16.

Caplan AJ, Cyr DM, and Douglas MG. (1993) Eukaryotic homologues of Escherichia coli dnaJ: a diverse protein family that functions with hsp70 stress proteins. Mol Biol Cell. **4:** 555-563.

Chaufour S, Mehlen P, and Arrigo AP (1996) Transient accumulation, phosphorylation and changes in the oligomerization of Hsp27 during retinoic acid-induced differentiation of HL-60 cells: possible role in the control of cellular growth and differentiation. Cell Stress Chaperones. **1:** 225-35.

Chavez Zobel AT, Loranger A, Marceau N, Thériault JR, Lambert H, and Landry J. (2003) Distinct chaperone mechanisms can delay the formation of aggresomes by the myopathy-causing R120G alphaB-crystallin mutant. Hum Mol Genet. **12:** 1609-1620.

Chiesa R, Gawinowicz-Kolks MA, Kleiman NJ, and Spector A. (1987) Identification of the specific phosphorylated serine in the bovine alpha crystallin A1 chain. Curr: Eye Res. **6:** 539-542.

Chiesa R, Noguera I, and Sredy J. (1997) Phosphorylation of HSP25 during lens cell differentiation. Exp Eye Res. **65:** 223-229.

Chiesi M, Longoni S, and Limbruno U. (1990) Cardiac alpha-crystallin. III. Involvement during heart ischemia. Mol. Cell Biochem. **97:** 129–136.

Chiti F and Dobson CM. (2006) Protein Misfolding, Functional Amyloid, and Human Disease Annual Review of Biochemistry. **75:** 333-366.

Choi J, Costa ML, Mermelstein CS, Chagas C, Holtzer S, and Holtzer H. (1990) MyoD converts primary dermal fibroblasts, chondroblasts, smooth muscle, and retinal pigmented epithelial cells into striated mononucleated myoblasts and multinucleated myotubes. Proc Natl Acad Sci. **87:** 7988–7992.

Chow SE, Lee RS, Shih SH, and Chen JK. (1998) Oxidized LDL promotes vascular endothelial cell pinocytosis via a prooxidation mechanism. FASEB J. **12:** 823–830.

Chowdary TK, Raman B, Ramakrishna T, and Rao CM. (2004)Mammalian Hsp22 is a heat-inducible small heat-shock protein with chaperone-like activity. Biochem J. **381:** 379-387.

Ciocca DR, Oesterreich S, Chamness GC, McGuire WL, and Fuqua SA. (1993) Biological and clinical implications of heat shock protein 27,000 (Hsp27): a review. J. Natl. Cancer Inst. **85:** 1558–1570.

Clark JI, and Huang QL. (1996) Modulation of the chaperone-like activity of bovine α-crystallin. Proc. Natl. Acad. Sci. **93:** 15185–15189.

Clegg CH, Linkhart TA, and Olwin BB. (1987) Growth factor control of skeletal muscle differentiation: commitment to terminal differentiation occurs in G1 phase and is repressed by fibroblast growth factor. J. Cell Biol. **105:** 949–956.

Cooper RN, Tajbakhsh S, Mouly V, Cossu G, Buckingham M, and Butler-Browne GS. (1999) In vivo satellite cell activation via Myf5 and MyoD in regenerating mouse skeletal muscle. . J Cell Sci. **112:** 2895-2901.

Costa ML, Escaleira R, Cataldo A, Oliveira F, and Mermelstein CS. (2004)Desmin: molecular interactions and putative functions of the muscle intermediate filament protein. Braz J Med Biol Res. **37:** 1819-30.

Cotto JJ, Kline M, and Morimoto RI. (1996). Activation of heat shock factor 1 DNA binding precedes stress-induced serine phosphorylation. Evidence for a multistep pathway of regulation. J Biol Chem. **271:** 3355–3358.

Craig EA, and Lindquist S. (1988). The heat-shock proteins. Annu Rev Genet. **22:** 631-677.

Cristau B, Schafer PH, and Pierce SK. (1994) Heat shock enhances antigen processing and accelerates the formation of compact class II alpha beta dimers. J Immunol. **152:** 1546–1556.

Cuenda A, and Cohen P. (1999) Stress-activated protein kinase-2/p38 and a rapamycin-sensitive pathway are required for C2C12 myogenesis. J Biol Chem. **274:** 4341–4346.

Currie RW, Karmazyn M, Kloc M, and Mailer K. (1988) Heat-shock response is associated with enhanced postischemic ventricular recovery. Circulat. Res. **63:** 543–549.

Dalakas MC, Park KY, Semino-Mora C, Lee HS, Sivakumar K, and Goldfarb LG. (2000) Desmin myopathy, a skeletal myopathy with cardiomyopathy caused by mutations in the desmin gene. N Engl J Med. **342:** 770–780.

Das BK, and Liang JJ-N. (1997) Detection and characterization of α-crystallin intermediate with maximal chaperone-like activity. Biochem. Biophys. Res. Commun. **236:** 370–374.

Datta SA, and Rao CM. (1999) Differential temperature-dependent chaperone-like activity of alphaA- and alphaB-crystallin homoaggregates. J Biol Chem. **274:** 34773–34778.

Datta SA, and Rao CM. (2000) Packing-induced conformational and functional changes in the subunits of alpha-crystallin. J Biol Chem. **275:** 41004-41010.

Davidson SM, and Morange M. (2000) Hsp25 and the p38 MAPK pathway are involved in differentiation of cardiomyocytes. Dev Biol. **218:** 146-160.

de Jong WW, Caspers GJ, and Leunissen JA. (1998) Genealogy of the alpha-crystallin--small heat-shock protein superfamily. Int J Biol Macromol. **22:** 151-162.

de Jong WW, Leunissen JA, and Voorter CE. (1993) Evolution of the alpha-crystallin/small heat-shock protein family. Mol Biol Evol. **10:** 103-26.

De Maio A. (1999) Heat shock proteins: facts, thoughts, and dreams. Shock. **11:** 1-12.

de Wit NJ, Verschuure P, Kappe G, King SM, de Jong WW, van Muijen GN, and Boelens, WC. (2004) Testis specific human small heat shock protein HSPB9 is a cancer/testis antigen, and potentially interacts with the dynein subunit TCTEL1. Eur J Cell Biol. **83:** 337–345.

Deato MD, Marr MT, Sottero T, Inouye C, Hu P, and Tjian R. (2008) MyoD targets TAF3/TRF3 to activate myogenin transcription. Mol Cell. **32:** 96-105.

den Engelsman J, Bennink EJ, Doerwald L, Onnekink C, Wunderink L, and Andley UP. (2004) Mimicking phosphorylation of the small heat-shock protein alphaB-crystallin recruits the F-box protein FBX4 to nuclear SC35 speckles. Eur. J. Biochem. **271:** 4195-4203.

den Engelsman J, Gerrits D, de Jong WW, Robbins J, Kato K and Boelens WC. (2005) Nuclear import of {alpha}B-crystallin is phosphorylation-dependent and hampered by hyperphosphorylation of the myopathy-related mutant R120G. J Biol Chem. **280:** 37139-37148.

den Engelsman J, Keijsers V, de Jong WW, and Boelens WC. (2003) The small heat-shock protein alpha B-crystallin promotes FBX4-dependent ubiquitination. J Biol Chem. **278:** 4699-704.

Deuerling E, and Bukau B. (2004) Chaperone-assisted folding of newly synthesized proteins in the cytosol. Crit Rev Biochem Mol Biol. **39:** 261-277.

Di Carlo A, De Mori R, Martelli F, Pompilio G, Capogrossi MC, and Germani A. (2004) Hypoxia inhibits myogenic differentiation through accelerated MyoD degradation. J Biol Chem. **279:** 16332-8.

DiDomenico BJ, Bugaisky GE, and Lindquist S. (1982). The heat shock response is self-regulated at both the transcriptional and posttranscriptional levels. Cell. **31**, 593-603.

Dill KA. (1990) Dominant forces in protein folding. Biochemistry. **29:** 7133-7155.

Dill KA, and Chan HS. (1997) From Levinthal to pathways to funnels. Nat Struct Biol. **4:** 10-9.

Dinner AR, Sali A, Smith LJ, Dobson CM, and Karplus M. (2000) Understanding protein folding via free-energy surfaces from theory and experiment. Trends Biochem Sci. **25:** 331-339.

Djabali K, de Nechaud B, Landon F, and Portier MM. (1997) AlphaB-crystallin interacts with intermediate filaments in response to stress. J Cell Sci. **110:** 2759–2769.

Djabali K, Piron G, de Néchaud B, and Portier MM. (1999) alphaB-crystallin interacts with cytoplasmic intermediate filament bundles during mitosis. Exp Cell Res. **253:** 649-662.

Dobson CM. (2004) Principles of protein folding, misfolding and aggregation. Semin Cell Dev Biol. **15:** 3-16.

Doran P, Gannon J, O'Connell K, and Ohlendieck K. (2007) Aging skeletal muscle shows a drastic increase in the small heat shock proteins RB-crystallin/HspB5 and cvHsp/HspB7. Eur J Cell Biol. **86:** 629–640.

Dougan DA, Mogk A, and Bukau B (2002) Protein folding and degradation in bacteria: to degrade or not to degrade? That is the question. Cell Mol Life Sci. **59:** 1607-1616.

Dubin RA, Gopal-Srivastava R, Wawrousek EF, and Piatigorsky J. (1991) Expression of the murine alpha B-crystallin gene in lens and skeletal muscle: identification of a muscle-preferred enhancer. Mol Cell Biol. **11:** 4340-4349.

Duverger O, Paslaru L, and Morange M. (2004) HSP25 is involved in two steps of the differentiation of PAM212 keratinocytes. J Biol Chem. **279:** 10252-10260.

Ecroyd H, and Carver JA. (2008) Crystallin proteins and amyloid fibrils. Cell Mol Life Sci. Sep 22.

Ellis J. (1987) Proteins as molecular chaperones. Nature. **328:** 378-379.

Ellis RJ, and van der Vies SM. (1991) Molecular chaperones. Annu Rev Biochem. **60:** 321–347.

Fagerholm PP, Philipson BT, and Lindstro¨m B, (1981) Normal human lens—the distribution of proteins. Exp. Eye Res. **33:** 615–620.

Fan CY, Lee S, and Cyr DM. (2003) Mechanisms for regulation of Hsp70 function by Hsp40. Cell Stress Chaperones. **8:** 309-316.

Favet N, Duverger O, Loones MT, Poliard A, Kellermann O, and Morange M. (2001) Overexpression of murine small heat shock protein HSP25 interferes with chondrocyte differentiation and decreases cell adhesion. Cell Death Differ. **8:** 603-613.

Fenton WA, Kashi Y, Furtak K, and Horwich AL. (1994) Residues in chaperonin GroEL required for polypeptide binding and release. Nature. **371:** 614-619.

Fernandes M, O'Brien T, and Lis JT. (1994) Structure and regulation of heat shock gene promoters. In *The biology of heat shock proteins and molecular chaperones.* 375–393. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Fernando P, Kelly JF, Balazsi K, Slack RS, and Megeney LA. (2002) Caspase 3 activity is required for skeletal muscle differentiation. Proc Natl Acad Sci. **99:** 11025-11030.

Ferrari G, and Mavilio F. (2002) Myogenic stem cells from the bone marrow: a therapeutic alternative for muscular dystrophy? Neuromuscul Disord. **1:** S7-S10.

Fink AL. (1999) Chaperone-mediated protein folding. Physiol Rev. **79:** 425-449.

Flaherty KM, McKay DB, Kabsch W, and Holmes KC. (1991) Similarity of the three-dimensional structures of actin and the ATPase fragment of a 70-kDa heat shock cognate protein. Proc Natl Acad Sci. **88:** 5041-5045.

Fontaine JM, Rest JS, Welsh MJ, and Benndorf R. (2003) The sperm outer dense fiber protein is the 10th member of the superfamily of mammalian small stress proteins. Cell Stress Chaperones. **8:** 62–69.

Freeman BC, and Morimoto RI. (1996) The human cytosolic molecular chaperones hsp90, hsp70 (hsc70) and hdj-1 have distinct roles in recognition of a non-native protein and protein refolding. EMBO J. **15:** 2969-2979.

Fujita Y, Ohto E, Katayama E, and Atomi Y. (2004) alphaB-Crystallin-coated MAP microtubule resists nocodazole and calcium-induced disassembly. J Cell Sci. **117:** 1719–1726.

Gangalum RK, Schibler MJ, and Bhat SP. (2004) Small heat shock protein alphaB-crystallin is part of cell cycle-dependent Golgi reorganization. J Biol Chem. **279:** 43374-43377.

Ganote CE, and Vander Heide RS. (1987) Cytoskeletal lesions in anoxic myocardial injury. A conventional and high-voltage electron-microscopic and immunofluorescence study. Am J Pathol. **129:** 327–344.

Garrido C, Bruey JM, Fromentin A, Hammann A, Arrigo AP, and Solary E. (1999) HSP27 inhibits cytochrome c-dependent activation of procaspase-9. FASEB J. **13:** 2061-2070.

Georgopoulos CP, Hendrix RW, Casjens SR, and Kaiser AD. (1973) Host participation in bacteriophage lambda head assembly. J Mol Biol. **76:** 45-60.

Gething MJ, and Sambrook J. (1992) Protein folding in the cell. Nature. **355:** 33-45.

Gething MJ, and Sambrook J. (1990) Transport and assembly processes in the endoplasmic reticulum. Semin Cell Biol. **1:** 65-72.

Giardina C, Perez-Riba M, and Lis JT. (1992) Promoter melting and TFIID complexes on *Drosophila* genes in vivo. Genes Dev. **6:** 2190–2200.

Glass JR, DeWitt RG, and Cress AE. (1985) Rapid loss of stress fibers in Chinese hamster ovary cells after hyperthermia. Cancer Res. **45:** 258–262.

Glover JR, and Lindquist S. (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. Cell. **94:** 73-82.

Goebel HH, Warlo I. (2000) Gene-related protein surplus myopathies. Mol Genet Metab. **71:** 267-75.

Goebel HH. (1995) Desmin-related neuromuscular disorders. Muscle Nerve. **18:** 1306-20.

Goebel HH. (2003) Congenital myopathies at their molecular dawning. Muscle Nerve. **27:** 527-48.

Goldfarb LG, Park KY, Cervenáková L, Gorokhova S, Lee HS, Vasconcelos O, Nagle JW, Semino-Mora C, Sivakumar K, and Dalakas MC. (1998) Missense mutations in desmin associated with familial cardiac and skeletal myopathy. Nat Genet. **19:** 402-403.

Goldfarb LG, Vicart P, Goebel HH, and Dalakas MC. (2004) Desmin myopathy. Brain. **127:** 723-734.

Golenhofen N, Ness W, Koob R., Htun P, Schaper W, and Drenckhahn D. (1998) Ischemia-induced phosphorylation and translocation of stress protein alpha B-crystallin to Z lines of myocardium. Am J Physiol. **274:** H1457–H1464.

Gredinger E, Gerber AN, Tamir Y, Tapscott SJ, and Bengal E. (1998) Mitogen-activated protein kinase pathway is involved in the differentiation of muscle cells. J Biol Chem. **273:** 10436-10444.

Grimm B, Ish-Shalom D, Even D, Glaczinski H, Ottersbach P, Ohad I, and Kloppstech K. (1989) The nuclear-coded chloroplast 22-kDa heat-shock protein of Chlamydomonas. Evidence for translocation into the organelle without a processing step. Eur J Biochem. **182:** 539-546.

Groenen PJ, Merck KB, de Jong WW, and Bloemendal H. (1994) Structure and modifications of the junior chaperone alpha-crystallin. From lens transparency to molecular pathology. Eur J Biochem. **225:** 1-19.

Gruvberger-Saal SK, and Parsons R. (2006) Is the small heat shock protein alphaB-crystallin an oncogene? J Clin Invest. **116:** 30-32.

Gu L, Abulimiti A, Li W, and Chang Z. (2002) Monodisperse Hsp16.3 nonamer exhibits dynamic dissociation and reassociation, with the nonamer dissociation prerequisite for chaperone-like activity. J Mol Biol. **319:** 517-526.

Guay J, Lambert H, Gingras-Breton G, Lavoie JN, Huot J, and Landry J. (1997) Regulation of actin filament dynamics by p38 map kinase-mediated phosphorylation of heat shock protein 27. J Cell Sci. **110:** 357–368.

Guo K, Wang J, Andrés V, Smith RC, and Walsh K. (1995) MyoD-induced expression of p21 inhibits cyclin-dependent kinase activity upon myocyte terminal differentiation. Mol Cell Biol. **15:** 3823-9.

Gupta R. (1990) Sequence and structural homology between a mouse t-complex protein TCP-1 and the 'chaperonin' family of bacterial (groEL, 60-65 kDa heat shock antigen) and eukaryotic proteins. Biochem Int. **20:** 833-841.

Gutsche I, Essen LO, and Baumeister W. (1999) Group II chaperonins: new TRiC(k)s and turns of a protein folding machine. J Mol Biol. **293:** 295-312.

Guttridge DC, Albanese C, Reuther JY, Pestell RG, and Baldwin AS Jr. (1999) NF-kappaB controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol. **19:** 5785-5799.

Guttridge DC, Mayo MW, Madrid LV, Wang CY, and Baldwin AS Jr. (2000) NF-kappaB-induced loss of MyoD messenger RNA: possible role in muscle decay and cachexia. Science. **289:** 2363-2366.

Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, Beach D, and Lassar AB. (1995) Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science. **267:** 1018-1021.

Haley DA, Horwitz J, and Stewart PL. (1998) The small heat-shock protein, *a*B-crystallin, has a variable quarternary structure. J Mol Biol. **277:** 27–35.

Han W, and Christen P. (2003) Mechanism of the targeting action of DnaJ in the DnaK molecular chaperone system. J Biol Chem. **278:** 19038-19043.

Harding JJ, and Dilley KJ. (1976). Structural proteins of the mammalian lens: A review with emphasis on changes in development, aging and cataract. Exp Eye Res. **22:** 1-73.

Hartl FU, and Hayer-Hartl M. (2002) Molecular chaperones in the cytosol: from nascent chain to folded protein. Science. **295:** 1852-1858.

Hartl FU, and Martin J. (1995) Molecular chaperones in cellular protein folding. Curr Opin Struct Biol. **5:** 92-102.

Hartl FU. (1996) Molecular chaperones in cellular protein folding. Nature. **381:** 571-579.

Haslbeck M, Franzmann T, Weinfurtner D, and Buchner J. (2005) Some like it hot: the structure and function of small heat-shock proteins. Nat Struct Mol Biol. **12:** 842-846.

Hasty P, Bradley A, Morris JH, Venuti JM, Olson EN, and Klein WH. (1993) Muscle deficiency and neonatal death in mice with a targeted mutation in the *myogenin* gene. Nature. **364:** 501– 506.

Hawke TJ, and Garry DJ. (2001) Myogenic satellite cells: physiology to molecular biology. J Appl Physiol. **91:** 534–551.

Hendershot LM, Valentine VA, Lee AS, Morris SW, and Shapiro DN. (1994) Localization of the gene encoding human BiP/GRP78, the endoplasmic reticulum cognate of the HSP70 family, to chromosome 9q34. Genomics. **20:** 281-284.

Hendrick JP, and Hartl FU. (1993) Molecular chaperone functions of Heat Shock Proteins. Annu Rev Biochem. **62:** 349-384.

Hoover HE, Thuerauf DJ, Martindale JJ, and Glembotski CC. (2000) alpha B-crystallin gene induction and phosphorylation by MKK6-activated p38. A potential role for alpha B-crystallin as a target of the p38 branch of the cardiac stress response. J Biol Chem. **275:** 23825–23833.

Horwitz J. (1992) Alpha-crystallin can function as a molecular chaperone. Proc Natl Acad Sci **89:** 10449-10453.

Horwitz J. (2003) Alpha-crystallin. Exp Eye Res. **76:** 145-153.

Hu Y, and Mivechi NF. (2003) HSF-1 interacts with Ral-binding protein 1 in a stress-responsive, multiprotein complex with HSP90 in vivo. J Biol Chem. **278:** 17299-17306.

Hunter T, and Pines J. (1994) Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell. **79:** 573-582.

Ikeda R, Yoshida K, Ushiyama M, Yamaguchi T, Iwashita K, Futagawa T, Shibayama Y, Oiso S, Takeda Y, Kariyazono H, Furukawa T, Nakamura K, Akiyama S, Inoue I, and Yamada K. (2006) The small heat shock protein alphaB-crystallin inhibits differentiation-induced caspase 3 activation and myogenic differentiation. Biol Pharm Bull. **29:** 1815-1819.

Inagaki N, Hayashi T, Arimura T, Koga Y, Takahashi M, Shibata H, Teraoka K, Chikamori T, Yamashina A, and Kimura A. (2006) Alpha B-crystallin mutation in dilated cardiomyopathy. Biochem Biophys Res Commun. **342:** 379-386.

Inaguma Y, Ito H, Iwamoto I, Saga S, and Kato K. (2001)AlphaB-crystallin phosphorylated at Ser-59 is localized in centrosomes and midbodies during mitosis. Eur J Cell Biol. **80:** 741-748.

Ingolia TD, and Craig EA. (1982) Four small Drosophila heat shock proteins are related to each other and to mammalian alpha-crystallin. Proc Natl Acad Sci. **79:** 2360-2364.

Inobe T, and Matouschek A. (2008) Protein targeting to ATP-dependent proteases. Curr Opin Struct Biol. **18:** 43-51.

Ito H, Kamei K, Iwamoto I, Inaguma Y, and Kato K. (2001a) Regulation of the levels of small heat-shock proteins during differentiation of C2C12 cells. Exp Cell Res. **266:** 213-221.

Ito H, Kamei K, Iwamoto I, Inaguma Y, Nohara D, and Kato K. (2001b) Phosphorylation-induced change of the oligomerization state of αB-crystallin. J Biol Chem. **276:** 5346–5352.

Ito H, Okamoto K, Nakayama H, Isobe T, and Kato K. (1997) Phosphorylation of alphaB-crystallin in response to various types of stress. J Biol Chem. **272:** 29934–29941.

Iwaki A, Nagano T, Nakagawa M, Iwaki T, and Fukumaki Y. (1997) Identification and characterization of the gene encoding a new member of the alpha-crystallin/small hsp family, closely linked to the alphaB-crystallin gene in a head-to-head manner. Genomics. **45:** 386-94.

Iwaki T, Iwaki A, Tateishi J, and Goldman JE. (1994) Sense and antisense modification of glial alpha B-crystallin production results in alterations of stress fiber formation and thermoresistance. J Cell Biol. **125:** 1385–1393.

Jaenicke R. (1991) Protein folding: local structures, domains, subunits, and assemblies. Biochemistry. **30:** 3147-3161.

Jaenicke R. (1995) Folding and association versus misfolding and aggregation of proteins. Philos Trans R Soc Lond B Biol Sci. **348:** 97–105.

Jakob U, Gaestel M, Engel K, Buchner J (1993) Small heat shock proteins are molecular chaperones. J Biol Chem. **268:** 1517-1520.

Jin JK, Whittaker R, Glassy MS, Barlow SB, Gottlieb RA, and Glembotski CC. (2008) Localization of phosphorylated alphaB-crystallin to heart mitochondria during ischemia-reperfusion. Am J Physiol Heart Circ Physiol. **294:** H337-H344.

Jirmanova I, Libelius R, Lundquist I, and Thesleff S. (1977) Protamine induced intracellular uptake of horseradish peroxidase and vacuolation in mouse skeletal muscle in vitro. Cell Tissue Res. **176:** 463–473.

Kamradt MC, Chen F, and Cryns VL. (2001) The small heat shock protein alpha B-crystallin negatively regulates cytochrome c- and caspase-8-dependent activation of caspase-3 by inhibiting its autoproteolytic maturation. J Biol Chem. **276:** 16059-16063.

Kamradt MC, Chen F, Sam S, and Cryns VL. (2002) The small heat shock protein alpha B-crystallin negatively regulates apoptosis during myogenic differentiation by inhibiting caspase-3 activation. J Biol Chem. **277:** 38731-38736.

Kamradt MC, Lu M, Werner ME, Kwan T, Chen F, Strohecker A, Oshita S, Wilkinson JC, Yu C, Oliver PG, Duckett CS, Buchsbaum DJ, LoBuglio AF, Jordan VC, and Cryns VL. (2005) The small heat shock protein alpha B-crystallin is a novel inhibitor of TRAIL-induced apoptosis that suppresses the activation of caspase-3. J Biol Chem. **280:** 11059–11066.

Kantorow M, and Piatigorsky J. (1998) Phosphorylations of alpha A- and alpha B-crystallin. Int J Biol Macromol. **22:** 307-314.

Kappe G, Leunissen JA, and de Jong WW. (2002) Evolution and diversity of prokaryotic small heat shock proteins. Prog Mol Subcell Biol. **28:** 1-17.

Kappe G, Verschuure P, Philipsen RL, Staalduinen AA, Van de Boogaart P, Boelens WC, and de Jong WW. (2001) Characterization of two novel human small heat shock proteins: protein kinase-related HspB8 and testis-specific HspB9. Biochim Biophys Acta. **1520:** 1-6.

Kassar-Duchossoy L, Gayraud-Morel B, Gomès D, Rocancourt D, Buckingham M, Shinin V, and Tajbakhsh S. (2004) Mrf4 determines skeletal muscle identity in Myf5:Myod double-mutant mice. Nature. **431:** 466-471.

Kato J, Matsushime H, Hiebert SW, Ewen ME, and Sherr CJ. (1993a) Direct binding of cyclin D to the retinoblastoma gene product (pRb) and pRb phosphorylation by the cyclin D-dependent kinase CDK4. Genes Dev. **7:** 331–342.

Kato K, Goto S, Hasegawa K, and Inaguma Y (1993b) Coinduction of two low-molecular-weight stress proteins, alpha B crystallin and HSP28, by heat or arsenite stress in human glioma cells. J Biochem. **114:** 640-647.

Kato K, Goto S, Hasegawa K, Shinohara H, and Inaguma Y. (1993c) Responses to heat shock of alpha B crystallin and HSP28 in U373 MG human glioma cells. Biochim Biophys Acta. **1175:** 257-262.

Kato K, Ito H, and Inaguma Y. (2002) Expression and phosphorylation of mammalian small heat shock proteins. Prog Mol Subcell Biol. **28:** 129-150.

Kato K, Ito H, Kamei K, Inaguma Y, Iwamoto I, and Saga S. (1998) Phosphorylation of alphaB-crystallin in mitotic cells and identification of enzymatic activities responsible for phosphorylation. J Biol Chem. **273:** 28346-28354.

Kato K, Shinohara H, Kurobe N, Goto S, Inaguma Y, and Ohshima K. (1991) Immunoreactive alpha A crystallin in rat non-lenticular tissues detected with a sensitive immunoassay method. Biochim Biophys Acta. **1080:** 173-180.

Kato K, Shinohara H, Goto S, Inaguma Y, Morishita R, Asano T. (1992) Copurification of small heat shock protein with alpha B crystallin from human skeletal muscle. J Biol Chem. **267:** 7718-25.

Kelley WL, and Georgopoulos C. (1992). Chaperones and protein folding. Curr Opin Cell Biol. **4:** 984-991.

Kitagawa M, Matsumura Y, and Tsuchido T. (2000) Small heat shock proteins, IbpA and IbpB, are involved in resistances to heat and superoxide stresses in Escherichia coli. FEMS Microbiol Lett. **184:** 165-171.

Kitagawa M, Wada C, Yoshioka S, and Yura T. (1991) Expression of ClpB, an analog of the ATP-dependent protease regulatory subunit in Escherichia coli, is controlled by a heat shock sigma factor (sigma 32). J Bacteriol **173:** 4247–4253.

Klein G, and Satre M. (1986) Kinetics of fluid-phase pinocytosis in Dictyostelium discoideum amoebae. Biochem Biophys Res Commun. **138:** 1146–1152.

Klemenz R, Fröhli E, Steiger RH, Schäfer R, and Aoyama A. (1991) Alpha B-crystallin is a small heat shock protein. Proc Natl Acad Sci. **88:** 3652-3656.

Klemenz R., Andres AC, Frohli E, Schafer R, and Aoyama A. (1993) Expression of the murine small heat shock protein hsp25 and *a*B-crystallin in the absence of stress. J Cell Biol. **120:** 639–645

Kline MP, and Morimoto RI. (1997). Repression of the heat shock factor 1 transcriptional activation domain is modulated by constitutive phosphorylation. Mol Cell Biol. **17:** 2107–2115.

Kotler DP, Tierney AR, Wang J, and Pierson RN, Jr. (1989) Magnitude of body-cell-mass depletion and the timing of death from wasting in AIDS. Am. J. Clin. Nutr. **50:** 444–447

Krief S, Faivre JF, Robert P, Le Douarin B, Brument-Larignon N, Lefrère I, Bouzyk MM, Anderson KM, Greller LD, Tobin FL, Souchet M, and Bril A. (1999) Identification and characterization of cvHsp. A novel human small stress protein selectively expressed in cardiovascular and insulin-sensitive tissues. J Biol Chem. **274:** 36592-36600.

Kumar LV, and Rao CM. (2000) Domain swapping in human αA- and αB- crystallins affects oligomerization and enhances chaperone-like activity. J Biol Chem. **275:** 22009 - 22013.

Kumar LV, Ramakrishna T, and Rao CM. (1999) Structural and functional consequences of the mutation of a conserved arginine residue in alphaA and alphaB crystallins. J Biol Chem. **274:** 24137–24141.

Kusumoto Y, Lomakin A, Teplow DB, and Benedek GB. (1998) Temperature dependence of amyloid β-protein fibrillization. Proc Natl Acad Sci. **95:** 12277–12282.

Lai BT, Chin NW, Stanek AE, Keh W, and Lanks KW. (1984) Quantitation and intracellular localization of the 85K heat shock protein by using monoclonal and polyclonal antibodies. Mol Cell Biol. **4:** 2802-2810.

Landry J, Bernier D, Chretien P, Nicole LM, Tanguay RM and Marceau N. (1982) Synthesis and degradation of heat shock proteins during development and decay of thermotolerance. Cancer Res. 42: 2457–2461.

Langen RC, Van Der Velden JL, Schols AM, Kelders MC, Wouters EF, and Janssen-Heininger YM. (2004) Tumor necrosis factor-alpha inhibits myogenic differentiation through MyoD protein destabilization. FASEB J. **18:** 227-237.

Laskey RA, Honda BM, Mills AD, and Finch JT. (1978) Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature. **275:** 416-420.

Laskowska E. (2007) Small heat shock proteins--role in apoptosis, cancerogenesis and diseases associated with protein aggregation. Postepy Biochem. **53:** 19-26.

Lassar AB, Skapek SX, and Novitch B. (1994) Regulatory mechanisms that coordinate skeletal muscle differentiation and cell cycle withdrawal. Curr Opin Cell Biol. **6:** 788–794.

Launay N, Goudeau B, Kato K, Vicart P, and Lilienbaum A. (2006) Cell signaling pathways to alphaB-crystallin following stresses of the cytoskeleton. Exp Cell Res. **312:** 3570–3584.

Lavoie JN, Hickey E, Weber LA, and Landry J. (1993) Modulation of actin microfilament dynamics and fluid phase pinocytosis by phosphorylation of heat shock protein 27. J Biol Chem. **268:** 24210-24214.

Lee S, Sowa ME, Watanabe YH, Sigler PB, Chiu W, Yoshida M, and Tsai F. (2003) The structure of ClpB: a molecular chaperone that rescues proteins from an aggregated state. Cell. **115:** 229-40.

Leopold PE, Montal M, and Onuchic JN. (1992) Protein folding funnels: a kinetic approach to the sequence-structure relationship. Proc Natl Acad Sci. **89:** 8721-8725.

Leustek T, Dalie B, Amir-Shapira D, Brot N, and Weissbach H. (1989) A member of the Hsp70 family is localized in mitochondria and resembles Escherichia coli DnaK. Proc Natl Acad Sci. **86:** 7805-7808.

Levinthal C. (1968) Are there pathways for protein folding? J. Chem. Phys. **65:** 44-45

Li GC, and Werb Z. (1982) Correlation between synthesis of heat shock proteins and development of thermotolerance in Chinese hamster fibroblasts. Proc Natl Acad Sci. **79:** 3218–3222.

Li H, and Capetanaki Y. (1993) Regulation of the mouse desmin gene: transactivated by MyoD, myogenin, MRF4 and Myf5. Nucleic Acids Research. **21:** 335-343.

Li LK, and Spector A. (1974) Circular dichroism and optical rotatory dispersion of the aggregates of purified polypeptides of alpha-crystallin. Exp Eye Res. **19:** 49-57.

Liberek K, Lewandowska A, and Zietkiewicz S. (2008) Chaperones in control of protein disaggregation.. EMBO J. **27:** 328-35.

Liberek K, Marszalek J, Ang D, Georgopoulos C, and Zylicz M. (1991) Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc Natl Acad Sci. **88:** 2874-2878.

Lin DI, Barbash O, Kumar KG, Weber JD, Harper JW, Klein-Szanto AJ, Rustgi A, Fuchs SY, and Diehl JA. (2006) Phosphorylation-dependent ubiquitination of cyclin D1 by the SCF (FBX4-alphaB crystallin) complex. Mol Cell. **24:** 355-366.

Lindquist S. (1981). Regulation of protein synthesis during heat shock. Nature. **293:** 311-14.

Lindquist S. (1986). The Heat Shock Response. Annual Review of Biochemistry. **55:** 151-91.

Lindquist S, and Craig EA. (1988) The heat-shock proteins. Annu Rev Genet. **22:** 631-77.

Lis J, and Wu C. (1993) Protein traffic on the heat shock promoter: Parking, stalling, and trucking along. Cell. **74:** 1–4.

Litt M, Kramer P, LaMorticella DM, Murphey W, Lovrien EW, and Weleber RG. (1998) Autosomal dominant congenital cataract associated with a missense mutation in the human alpha crystallin gene CRYAA. Hum Mol Genet. **7:** 471-474.

Liu JP, Schlosser R, Ma WY, Dong Z, Feng H, Lui L, Huang XQ, Liu Y, and Li DW. (2004) Human alphaA- and alphaB-crystallins prevent UVA-induced apoptosis through regulation of PKCalpha, RAF/MEK/ERK and AKT signaling pathways. Exp Eye Res. **79:** 393-403.

Lowe J, McDermott H, Pike I, Spendlove I, Landon M, and Mayer RJ. (1992) alpha B crystallin expression in non-lenticular tissues and selective presence in ubiquitinated inclusion bodies in human disease. J Pathol. **166:** 61-68.

Lum R, Tkach JM, Vierling E, and Glover JR. (2004) Evidence for an unfolding/threading mechanism for protein disaggregation by Saccharomyces cerevisiae Hsp104. J Biol Chem **279:** 29139-29146.

Lundberg IE, and Dastmalchi M. (2002) Possible pathogenic mechanisms in in-flammatory myopathies. Rheum Dis Clin North Am **28:** 799–822.

Macario AJ, Lange M, Ahring BK, and De Macario EC. (1999) Stress genes and proteins in the archaea. Microbiol Mol Biol Rev. **63:** 923-967

Mackay DS, Andley UP, and Shiels A. (2003) Cell death triggered by a novel mutation in the alphaA-crystallin gene underlies autosomal dominant cataract linked to chromosome 21q. Eur J Hum Genet. **11:** 784-793.

Magin TM, Schröder R, Leitgeb S, Wanninger F, Zatloukal K, Grund C, and Melton DW. (1998) Lessons from keratin 18 knockout mice: formation of novel keratin filaments, secondary loss of keratin 7 and accumulation of liver-specific keratin 8-positive aggregates. J Cell Biol. **140:** 1441-1451.

Maione R, and Amati P. (1997) Interdependence between muscle differentiation and cell-cycle control. Biochim. Biophys Acta Rev Cancer. **332:** M19–M30.

Maloyan A, Sanbe A, Osinska H, Westfall M, Robinson D, Imahashi K, Murphy E, and Robbins J. (2005) Mitochondrial dysfunction and apoptosis underlie the pathogenic process in alpha-B-crystallin desmin-related cardiomyopathy. Circulation. **112:** 3451-3461.

Mao YW, Liu JP, Xiang H, and Li DW. (2004) Human alphaA- and alphaB-crystallins bind to Bax and Bcl-X(S) to sequester their translocation during staurosporine-induced apoptosis. Cell Death Differ. **11:** 512-526.

Martin J, and Hartl FU. (1997) The effect of macromolecular crowding on chaperonin-mediated protein folding Proc Natl Acad Sci. **94:** 1107-1112

Martin J, Horwich AL, and Hartl FU. (1992) Prevention of protein denaturation under heat stress by the chaperonin Hsp60. Science. **258:** 995-998.

Martin J, Langer T, Boteva R, Schramel A, Horwich AL, and Hartl FU. (1991) Chaperonin-mediated protein folding at the surface of groEL through a 'molten globule'-like intermediate. Nature. **352:** 36-42.

Martin SS, and Leder P. (2001) Human MCF10A mammary epithelial cells undergo apoptosis following actin depolymerization that is independent of attachment and rescued by Bcl-2. Mol Cell Biol. **21:** 6529–6536.

Mason PB, Jr. and Lis JT. (1997) Cooperative and competitive protein interactions at the hsp70 promoter. J Biol Chem. **272:** 33227–33233.

Mazzarella RA, and Green M. (1987) ERp99, an abundant, conserved glycoprotein of the endoplasmic reticulum, is homologous to the 90 kDa heat shock protein (hsp90) and the 94-kDa glucose regulated protein (grp94). J Biol Chem. **262:** 8875–8883.

McClellan AJ, Xia Y, Deutschbauer AM, Davis RW, Gerstein M, and Frydman J. (2007) Diverse cellular functions of the Hsp90 molecular chaperone uncovered using systems approaches. Cell. **131:** 121-135.

McMillan DR, Xiao X, Shao L, Graves K, and Benjamin IJ. (1998) Targeted disruption of heat shock transcription factor1 abolishes thermotolerance and protection against heat-inducible apoptosis. J. Biol. Chem. **273:** 7523–7528.

Megeney LA, Kablar B, Garrett K, Anderson JE, and Rudnicki MA. (1996) MyoD is required for myogenic stem cell function in adult skeletal muscle. Genes Dev. **10:** 1173-1183.

Mehlen P, and Arrigo AP. (1994) The serum-induced phosphorylation of mammalian hsp27 correlates with changes in its intracellular localization and levels of oligomerization. Eur J Biochem. **221:** 327-334.

Mehlen P, Preville X, Chareyron P, Briolay J, Klemenz R, and Arrigo AP. (1995) Constitutive expression of human hsp27, Drosophila hsp27, or human alphaB-crystallin confers resistance to TNF- and oxidative stress-induced cytotoxicity in stably transfected murine L929 fibroblasts. J Immunol. **154:** 363-374.

Merck KB, Groenen PJ, Voorter CE, de Haard-Hoekman WA, Horwitz J, Bloemendal H, and de Jong WW. (1993) Structural and functional similarities of bovine alpha-crystallin and mouse small heat-shock protein. A family of chaperones. J Biol Chem. **268:** 1046-1052.

Miron T, Vancompernolle K, Vandekerckhove J, Wilchek M, and Geiger B. (1991) A 25-kD inhibitor of actin polymerization is a low molecular mass heat shock protein. J Cell Biol. **114:** 255–261.

Misselwitz B, Staeck O, and Rapoport TA. (1998) J proteins catalytically activate Hsp70 molecules to trap a wide range of peptide sequences. Mol Cell. **2:** 593-603.

Mogk A, Mayer MP, and Deuerling E. (2002) Mechanisms of protein folding: molecular chaperones and their application in biotechnology. Chembiochem. **3:** 807-814.

Molkentin JD, Black BL, Martin JF, and Olson EN. (1995) Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH proteins. Cell. **83:**1125–1136.

Morimoto RI, Tissieres A, and Georgopoulos C. (1990) The stress response, function of the proteins, and perspectives. In *Stress proteins in biology and medicine*, 1–36. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Morimoto RI. (1993) Cells in stress: transcriptional activation of heat shock genes. Science. **259:** 1409–1410.

Morimoto RI. (1998) Regulation of the heat shock transcriptional response: cross talk between a family of heat shock factors, molecular chaperones, and negative regulators. Genes Dev. **12:** 3788-3796.

Morrison LE, Hoover HE, Thuerauf DJ, and Glembotski CC. (2003) Mimicking phosphorylation of alphaB-crystallin on serine-59 is necessary and sufficient to provide maximal protection of cardiac myocytes from apoptosis. Circ Res. **92:** 203–211.

Morrison LE, Whittaker RJ, Klepper RE, Wawrousek EF, and Glembotski CC. (2004) Roles for alphaB-crystallin and HSPB2 in protecting the myocardium from ischemia-reperfusion-induced damage in a KO mouse model. Am J Physiol Heart Circ Physiol. **286:** H847-H8455.

Mosser DD, Duchaine J, and Massie B. (1993) The DNA-binding activity of the human heat shock transcription factor is regulated in vivo by hsp70. Mol Cell Biol. **13:** 5427–5438.

Muchowski PJ, and Clark JI. (1998) ATP-enhanced molecular chaperone functions of the small heat shock protein human alphaB crystallin. Proc Natl Acad Sci. **95:** 1004-1009.

Muchowski PJ. (2002) Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? Neuron. **35:** 9-12.

Mukasa T, Momoi T, and Momoi MY. (1999) Activation of caspase-3 apoptotic pathways in skeletal muscle fibers in laminin alpha2-deficient mice. Biochem Biophys Res Commun. **260:** 139-142.

Munoz-Marmol AM, Strasser G, Isamat M, Coulombe PA, Yang Y, Roca X, Vela E, Mate JL, Coll J, Fernández-Figueras MT, Navas-Palacios JJ, Ariza A, and Fuchs E. (1998) A dysfunctional desmin mutation in a patient with severe generalized myopathy. Proc Natl Acad Sci. **95:** 11312-11317.

Munro S, and Pelham HR. (1986) An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell. **46:** 291-300.

Nakai A, and Morimoto RI. (1993) Characterization of a novel chicken heat shock transcription factor, heat shock factor 3, suggests a new regulatory pathway. Mol Cell Biol. **13:** 1983–1997.

Nakase I, Niwa M, Takeuchi T, Sonomura K, Kawabata N, Koike Y, Takehashi M, Tanaka S, Ueda K, Simpson JC, Jones AT, Sugiura Y, and Futaki S. (2004) Cellular uptake of arginine-rich peptides: roles for macropinocytosis and actin rearrangement. Mol Ther. **10:** 1011–1022.

Nedellec P, Edling Y, Perret E, Fardeau M, and Vicart P. (2002) Glucocorticoid treatment induces expression of small heat shock proteins in human satellite cell populations: consequences for a desmin-related myopathy involving the R120G alpha B-crystallin mutation. Neuromuscul Disord. **12:** 457-465.

Nefti O, Grongnet JF, and David JC. (2005) Overexpression of alphaB crystallin in the gastrointestinal tract of the newborn piglet after hypoxia. Shock. **24:** 455-461.

Neufer PD, and Benjamin IJ. (1996) Differential expression of B-crystallin and Hsp27 in skeletal muscle during continuous contractile activity. Relationship to myogenic regulatory factors. J Biol Chem. **271:** 24089-24095.

Nguyen AN, and Shiozaki K. (1999) Heat-shock-induced activation of stress MAP kinase is regulated by threonine- and tyrosine-specific phosphatases. Genes Dev. **13:** 1653-1663.

Nicholl ID, and Quinlan RA. (1994) Chaperone activity of alpha-crystallins modulates intermediate filament assembly. EMBO J. **13:** 945–953.

Nishida S, Hiruma S, and Hashimoto S. (1987) Immunohistochemical change of actin in experimental myocardial ischemia. Its usefulness to detect very early myocardial damages. Histol. Histopathol. **2:** 417–428.

Nishikawa S, Brodsky JL, and Nakatsukasa K. (2005) Roles of molecular chaperones in endoplasmic reticulum (ER) quality control and ER-associated degradation (ERAD). J Biochem. **137:** 551-555.

Nunez G, Benedict MA, Hu Y, and Inohara N. (1998) Caspases: the proteases of the apoptotic pathway. Oncogene. **17:** 3237-3245.

Nurse P. (1994) Ordering S phase and M phase in the cell cycle. Cell **79:** 547–550.

Pace CN, Shirley BA, McNutt M, and Gajiwala K. (1996) Forces contributing to the conformational stability of proteins. FASEB J. **10:** 75-83.

Pace CN. (1995). Evaluating contribution of hydrogen bonding and hydrophobic bonding to protein folding. Methods Enzymol. **259**, 538-554.

Palmer WG, and Papaconstantinou J. (1969) Aging of alpha-crystallins during development of the lens. Proc Natl Acad Sci. **64:** 404-410.

Palmisano DV, Groth-Vasselli B, Farnsworth PN, and Reddy MC. (1995) Interaction of ATP and lens alpha crystallin characterized by equilibrium binding studies and intrinsic tryptophan fluorescence spectroscopy. Biochim Biophys Acta. **1246:** 91-97.

Parcellier A, Schmitt E, Gurbuxani S, Seigneurin-Berny D, Pance A, Chantôme A, Plenchette S, Khochbin S, Solary E, and Garrido C (2003) HSP27 is a ubiquitin-binding protein involved in I-kappaBalpha proteasomal degradation. . Mol Cell Biol. **23:** 5790-5802.

Park KJ, Gaynor RB, and Kwak YT. (2003) Heat shock protein 27 association with the I kappa B kinase complex regulates tumor necrosis factor alpha-induced NF-kappa B activation. J Biol Chem. **278:** 35272-35278.

Parsell DA, and Lindquist S. (1993). The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. Annu Rev Genet. **27:** 437–496.

Parsell DA, and Sauer RT. (1989) Induction of a heat shock-like response by unfolded protein in Escherichia coli: dependence on protein level not protein degradation. Genes Dev. **3:** 1226-1232.

Parsell DA, Kowal AS, Singer MA, and Lindquist S. (1994) Protein disaggregation mediated by heat-shock protein Hsp 104. Nature. **372:** 475-478.

Pasta SY, Raman B, Ramakrishna T and Rao CM. (2002) Role of C-terminal extensions of alpha-crystallins: swapping the C-terminal extension of αA-crystallin to αB-crystallin results in enhanced chaperone activity. J Biol Chem. **277:** 45821-45828.

Pasta SY, Raman B, Ramakrishna T and Rao CM. (2004) The IXI/V motif in the C-terminal extension of α-crystallins: alternative interactions and oligomeric assemblies. Mol Vis. **10:** 655-662.

Pasta SY, Raman B, Ramakrishna T and Rao CM. (2003) Role of the conserved SRLFDQFFG region of alpha-crystallin, a small heat shock protein. Effect on oligomeric size, subunit exchange, and chaperone-like activity. J Biol Chem. **278:** 51159-51166.

Pearl LH, and Prodromou C. (2000) Structure and in vivo function of Hsp90. Curr Opin Struct Biol **10:** 46-51.

Pelham HRB. (1982) A regulatory upstream promoter element in the Drosophila hspT0 heat shock gene. Cell. **30:** 517-528.

Perng MD, Cairns L, van den IJssel P, Prescott A, Hutcheson AM, and Quinlan RA. (1999) Intermediate filament interactions can be altered by HSP27 and alphaB-crystallin. J Cell Sci. **112:** 2099–2112.

Perng MD, Muchowski PJ, van Den IJssel P, Wu GJ, Hutcheson AM, Clark JI, and Quinlan RA. (1999) The cardiomyopathy and lens cataract mutation in alphaB-crystallin alters its protein structure, chaperone activity, and interaction with intermediate filaments in vitro. J Biol Chem. **274:** 33235-33243.

Perng MD, Wen SF, van den IJssel P, Prescott AR, and Quinlan RA. (2004) Desmin aggregate formation by R120G alphaB-crystallin is caused by altered filament interactions and is dependent upon network status in cells. Mol Biol Cell. **15:** 2335-2346.

Petersen R, and Lindquist S. (1988) The Drosophila hsp70 message is rapidly degraded at normal temperatures and stabilized by heat shock. Gene. **10:**161-168.

Pierpaoli EV, Sandmeier E, Baici A, Schönfeld HJ, Gisler S, and Christen P. (1997) The power stroke of the DnaK/DnaJ/GrpE molecular chaperone system. J Mol Biol. **269:** 757-768.

Pinto M, Morange M, and Bensaude O. (1991) Denaturation of proteins during heat shock. In vivo recovery of solubility and activity of reporter enzymes. J Biol Chem. **266:** 13941-13946.

Plesofsky-Vig N, Vig J, and Brambl R. (1992) Phylogeny of the alpha-crystallin-related heat-shock proteins. J Mol Evol. **35:** 537-545.

Qiu XB, Shao YM, Miao S, and Wang L. (2006) The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cell Mol Life Sci. **63:** 2560-2570.

Raingeaud J, Whitmarsh AJ, Barrett T, Dérijard B, and Davis RJ. (1996) MKK3- and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway. Mol Cell Biol. **16:** 1247-1255.

Rallu M, Loones M, Lallemand Y, Morimoto RI, Morange M, and Mezger V. (1997). Function and regulation of heat shock factor 2 during mouse embryogenesis. Proc Natl Acad Sci. **94:** 2392–2397.

Raman B, and Rao CM. (1994) Chaperone-like activity and quaternary structure of alpha-crystallin. J Biol Chem. **269:** 27264–27268.

Raman B, and Rao CM. (1997) Chaperone-like activity and temperature-induced structural changes of alpha-crystallin. J Biol Chem. **272:** 23559-23564.

Raman B, Ramakrishna T, and Rao CM. (1995) Temperature dependent chaperone-like activity of alpha-crystallin. FEBS Lett. **365:** 133-136.

Rao CM, Raman B, Ramakrishna T, Rajaraman K, Ghosh D, Datta S, Trivedi VD, and Sukhaswami MB. (1998) Structural perturbation of alpha-crystallin and its chaperone-like activity. Int J Biol Macromol. **22:** 271-281.

Rao CM, Zigler SJ, Raman B. (1993). Differential protective ability of alpha-crystallin towards thermal and photoaggregation of gamma-crystallin. Invest. Ophthalmol. Vis. Sci. **34**, 988

Rao SS, Chu C, and Kohtz DS. (1994) Ectopic expression of cyclin D1 prevents activation of gene transcription by myogenic basic helix-loop-helix regulators. Mol Cell Biol. **14:** 5259–5267.

Reichard EA, Ball WS Jr, and Bove KE. (1996) Alexander disease: a case report and review of the literature. Pediatr Pathol Lab Med. **16:** 327-343.

Resnitzky D, Gossen M, Bujard H, and Reed SI. (1994) Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. Mol Cell Biol. **14:** 1669–1679.

Riggs DL, Cox MB, Cheung-Flynn J, Prapapanich V, Carrigan PE, and Smith DF. (2004) Functional specificity of co-chaperone interactions with Hsp90 client proteins. Crit Rev Biochem Mol Biol. **39:** 279-295.

Ritossa F. (1962). New puffing pattern induced by temperature shock and DNP in Drosophila. Experientia. **18:** 571-573.

Rose GD, and Wolfenden R. (1993) Hydrogen bonding, hydrophobicity, packing, and protein folding. Annu Rev Biophys Biomol Struct. **22:** 381-415.

Roseman AM, Chen S, White H, Braig K, and Saibil HR. (1996) The chaperonin ATPase cycle: mechanism of allosteric switching and movements of substrate-binding domains in GroEL. Cell. **87:** 241-251.

Rougvie AE, and Lis JT. (1988) The RNA polymerase II molecule at the 5' end of the uninduced hsp70 gene of D. melanogaster is transcriptionally engaged. Cell. **54:** 795–804.

Rudnicki MA, Schnegelsberg PNJ, Stead RH, Braun T, Arnold HH, and Jaenisch R. (1993) MyoD or Myf-5 is required for the formation of skeletal muscle. Cell. **75:** 1351– 1359.

Sabourin LA, Girgis-Gabardo A, Seale P, Asakura A, and Rudnicki MA. (1999) Reduced differentiation potential of primary MyoD-/- myogenic cells derived from adult skeletal muscle. J Cell Biol. **144:** 631-643.

Saito N. (1989) Principles of protein architecture. Adv Biophys. **25:** 95-132.

Sanbe A, Osinska H, Saffitz JE, Glabe CG, Kayed R, Maloyan A, and Robbins J. (2004) Desmin-related cardiomyopathy in transgenic mice: a cardiac amyloidosis. Proc Natl Acad Sci. **101:** 10132-10136.

Sanchez Y, and Lindquist SL. (1990) HSP104 required for induced thermotolerance. Science. **248:** 1112-1115.

Santhiya ST, Soker T, Klopp N, Illig T, Prakash MV, Selvaraj B, Gopinath PM, and Graw J. (2006) Identification of a novel, putative cataract-causing allele in CRYAA (G98R) in an Indian family. Mol Vis. **12:** 768-773.

Sarge KD, Murphy SP, and Morimoto RI. (1993). Activation of heat shock gene transcription by heat shock factor1 involves oligomerization, acquisition of DNA-binding activity, and nuclear localization and can occur in the absence of stress. Mol Cell Biol. **13:** 1392-1407.

Sartorelli V, and Caretti G. (2005) Mechanisms underlying the transcriptional regulation of skeletal myogenesis. Curr Opin Genet Dev. **15:** 528–535.

Satyal SH, Chen D, Fox SG, Kramer JM, and Morimoto RI. (1998). Negative regulation of the heat shock transcriptional response by HSBP1. Genes Dev. **12:** 1962-1974.

Sauer RT, Bolon DN, Burton BM, Burton RE, Flynn JM, Grant RA, Hersch GL, Joshi SA, Kenniston JA, Levchenko I, Neher SB, Oakes ES, Siddiqui SM, Wah DA, and Baker TA. (2004). Sculpting the proteome with AAA(+) proteases and disassembly machines. Cell. **119:** 9-18.

Scheibel T, Siegmund HI, Jaenicke R, Ganz P, Lilie H, and Buchner J. (1999) The charged region of Hsp90 modulates the function of the N-terminal domain. Proc Natl Acad Sci. **96:** 1297-1302.

Scheufler C, Brinker A, Bourenkov G, Pegoraro S, Moroder L, Bartunik H, Hartl FU, and Moarefi I. (2000) Structure of TPR domain-peptide complexes: critical elements in the assembly of the Hsp70-Hsp90 multichaperone machine. Cell. **101:** 199-210.

Schirmer EC, Glover JR, Singer MA, and Lindquist S. (1996) HSP100/Clp proteins: a common mechanism explains diverse functions. Trends Biochem Sci. **21:** 289–296.

Schmid D, Baici A, Gehring H, and Christen P. (1994) Kinetics of molecular chaperone action. Science. **263:** 971-973.

Schneider HC, Berthold J, Bauer MF, Dietmeier K, Guiard B, Brunner M, and Neupert W. (1994) Mitochondrial Hsp70/MIM44 complex facilitates protein import. Nature. **371:** 768-774.

Schols AM, Slangen J, Volovics L, and Wouters EF. (1998) Weight loss is a reversible factor in the prognosis of chronic obstructive pulmonary disease. Am J Respir Crit Care Med. **157:** 1791–1797.

Selcen D. (2008) Myofibrillar myopathies. Curr Opin Neurol. **21:** 585.

Sharma M, Pampinella F, Nemes C, Benharouga M, So J, Du K, Bache KG, Papsin B, Zerangue N, Stenmark H, and Lukacs GL. (2004). Misfolding diverts CFTR from recycling to degradation: quality control at early endosomes. J. Cell Biol. **164:** 923–933.

Sherr CJ. (1994) G1 phase progression: cycling on cue. Cell.**79:** 551–555.

Shi Y, Kroeger PE, and Morimoto RI. (1995) The carboxyl-terminal transactivation domain of heat shock factor 1 is negatively regulated and stress responsive. Mol Cell Biol. **15:** 4309-4318.

Shi Y, Mosser DD, and Morimoto RI. (1998) Molecular chaperones as HSF1-specific transcriptional repressors. Genes and Dev. **12:** 654–666.

Siezen RJ, and Argos P. (1983) Structural homology of lens crystallins. III. Secondary structure estimation from circular dichroism and prediction from amino acid sequences. Biochim Biophys Acta. **748:** 56-67.

Siezen RJ, Bindels JG, and Hoenders HJ. (1978) The quaternary structure of bovine alpha-crystallin. Size and charge microheterogeneity: more than 1000 different hybrids? Eur J Biochem. **91:** 387-396.

Singh BN, Rao KS, Ramakrishna T, Rangaraj N, and Rao CM. (2007) Association of alphaB-crystallin, a small heat shock protein, with actin: role in modulating actin filament dynamics in vivo. J Mol Biol. **366:** 756-767.

Singh D, Raman B, Ramakrishna T, and Rao CM. (2006) The cataract-causing mutation G98R in human alphaA-crystallin leads to folding defects and loss of chaperone activity. Mol Vis. **12:** 1372-1379.

Sitterding SM, Wiseman WR, Schiller CL, Luan C, Chen F, Moyano JV, Watkin WG, Wiley EL, Cryns VL, Diaz LK. (2008) AlphaB-crystallin: a novel marker of invasive basal-like and metaplastic breast carcinomas. Ann Diagn Pathol. **12:** 33-40.

Skapek SX, Rhee J, Spicer DB, and Lassar AB. (1995) Inhibition of myogenic differentiation in proliferating myoblasts by cyclin D1-dependent ki nase. Science. **267:** 1022–1024.

Slepenkov SV, and Witt SN. (1998) Peptide-induced conformational changes in the molecular chaperone DnaK. Biochemistry. **37:** 16749-16756.

Smith JB, Sun Y, Smith DL, and Green B. (1992) Identification of the posttranslational modifications of bovine lens alphaB-crystallins by mass spectrometry. Protein Sci. **1:** 601–608.

Somasundaram T, and Bhat SP. (2000) Canonical heat shock element in the alpha B-crystallin gene shows tissue-specific and developmentally controlled interactions with heat shock factor. J Biol Chem. **275:** 17154-17159.

Spector A, Li LK, Augusteyn RC, Schneider A, and Freund T. (1971) Crystallin. The isolation and characterization of distinct macromolecular fractions. Biochem J. **124:** 337-343.

Srinivas V, Raman B, Rao KS, Ramakrishna T, and Rao CM. (2003) Structural perturbation and enhancement of the chaperone-like activity of alpha-crystallin by arginine hydrochloride. Protein Sci. **12:** 1262-1270.

Stahl J, Wobus AM, Ihrig S, Lutsch G, and Bielka H. (1992) The small heat shock protein hsp25 is accumulated in P19 embryonal carcinoma cells and embryonic stem cells of line BLC6 during differentiation. Differentiation. **51:** 33-37.

Sugiyama Y, Suzuki A, Kishikawa M, Akutsu R, Hirose T, Waye MM, Tsui SK, Yoshida S, and Ohno S. (2000) Muscle develops a specific form of small heat shock protein complex composed of MKBP/HSPB2 and HSPB3 during myogenic differentiation. J Biol Chem. **275:** 1095-1104.

Sun Y, and MacRae TH. (2005) The small heat shock proteins and their role in human disease. FEBS J. **272:** 2613-2627.

Suzuki A, Sugiyama Y, Hayashi Y, Nyu-i N, Yoshida M, Nonaka I, Ishiura S, Arahata K, and Ohno S. (1998) MKBP, a novel member of the small heat shock protein family, binds and activates the myotonic dystrophy protein kinase. J Cell Biol. **140:** 1113-1124.

Szabo A, Langer T, Schröder H, Flanagan J, Bukau B, and Hartl FU. (1994) The ATP hydrolysis-dependent reaction cycle of the Escherichia coli Hsp70 system DnaK, DnaJ, and GrpE. Proc Natl Acad Sci. **91:** 10345-10349.

Tanabe M, Kawazoe Y, Takeda S, Morimoto RI, Nagata K, and Nakai A. (1998) Disruption of the HSF3 gene results in the severe reduction of heat shock gene expression and loss of thermotolerance. EMBO J. **17:** 1750–1758.

Tannous P, Zhu H, Johnstone JL, Shelton JM, Rajasekaran NS, Benjamin IJ, Nguyen L, Gerard RD, Levine B, Rothermel BA, and Hill JA. (2008) Autophagy is an adaptive response in desmin-related cardiomyopathy. Proc Natl Acad Sci. **105:** 9745-50.

Theriot JA, and Mitchison TJ. (1991) Actin microfilament dynamics in locomoting cells. Nature. **352:** 126–131.

Thompson MA, Hawkins JW, and Piatigorsky J. (1987) Complete nucleotide sequence of the chicken alpha A-crystallin gene and its 5' flanking region. Gene. **56:** 173-184.

Thornberry NA, and Lazebnik Y. (1998) Caspases: Enemies Within. Science. **281:** 1312 – 1316

Tissières A, Mitchell HK, and Tracy UM. (1974) Protein synthesis in salivary glands of Drosophila melanogaster: relation to chromosome puffs. J Mol Biol. **84:** 389-398.

Tumminia SJ, and Russell P. (1994) Alpha B-crystallin accumulation in human astroglioma cell line U373MG is stress-dependent and phosphorylation-independent. J Biochem. **116:** 973-979.

van den Oetelaar PJ, van Someren PF, Thomson JA, Siezen RJ, and Hoenders HJ (1990) A dynamic quaternary structure of bovine alpha-crystallin as indicated from intermolecular exchange of subunits. . Biochemistry. **29:** 3488-3493.

Van Deurs B, and Nilausen K. (1982) Pinocytosis in mouse L-fibroblasts: ultrastructural evidence for a direct membrane shuttle between the plasma membrane and the lysosomal compartment. J Cell Biol. **94:** 279–286.

Van Kleef SM, Willems-Thijssen W, and Hoenders HJ. (1976) Intracellular degradation and deamidation of alpha-crystallin subunits. Eur J Biochem. **66:** 477-483.

van Montfort RL, Basha E, Friedrich KL, Slingsby C, and Vierling E. (2001) Crystal structure and assembly of a eukaryotic small heat shock protein. Nat Struct Biol. **8:** 1025-1030.

Vary TC, Owens EL, Beers JK, Verner K, and Cooney RN. (1996) Sepsis inhibits synthesis of myofibrillar and sarcoplasmic proteins: modulation by interleukin-1 receptor antagonist. Shock. **6:** 13–18

Verschuure P, Croes Y, van den IJssel PR, Quinlan RA, de Jong WW, and Boelens WC. (2002) Translocation of small heat shock proteins to the actin cytoskeleton upon proteasomal inhibition. J Mol Cell Cardiol. **34:** 117-128.

Verschuure P, Tatard C, Boelens WC, Grongnet JF, David JC. (2003) Expression of small heat shock proteins HspB2, HspB8, Hsp20 and cvHsp in different tissues of the perinatal developing pig. Eur J Cell Biol. **82:** 523-30.

Vicart P, Caron A, Guicheney P, Li Z, Prévost MC, Faure A, Chateau D, Chapon F, Tomé F, Dupret JM, Paulin D, and Fardeau M. (1998) A missense mutation in the alphaB-crystallin chaperone gene causes a desmin-related myopathy. Nat Genet. **20:** 92-95.

Voellmy R. (1994) Transduction of the stress signal and mechanisms of transcriptional regulation of heat shock/stress protein gene expression in higher eukaryotes. Crit Rev Eukaryot Gene Expr. **4:** 357–401.

Vos MJ, Hageman J, Carra S, and Kampinga HH. (2008) Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. Biochemistry. **47:** 7001-7011.

Voss OH, Batra S, Kolattukudy SJ, Gonzalez-Mejia ME, Smith JB, and Doseff AI. (2007) Binding of caspase-3 prodomain to heat shock protein 27 regulates monocyte apoptosis by inhibiting caspase-3 proteolytic activation. J Biol Chem. **282:** 25088-25099.

Wagner R, Rosenberg M, and Estensen R. (1971) Endocytosis in Chang liver cells. Quantitation by sucrose-3H uptake and inhibition by cytochalasin B. J Cell Biol. **50:** 804–817.

Walsh K, and Perlman H. (1997) Cell cycle exit upon myogenic differentiation. Curr Opin Genet Dev. **7:** 597–602.

Walsh K. (1997) Coordinate regulation of cell cycle and apoptosis during myogenesis. Prog Cell Cycle Res. **3:** 53-58.

Wang K, and Spector A. (1995) Alpha-crystallin can act as a chaperone under conditions of oxidative stress. Invest Ophthalmol Vis Sci. **36:** 311–321.

Wang K, and Spector A. (1996) alpha-crystallin stabilizes actin filaments and prevents cytochalasin-induced depolymerization in a phosphorylation-dependent manner. Eur J Biochem. **242:** 56-66.

Wang W, Zhang W, Han Y, Chen J, Wang Y, Zhang Z, and Hui R. (2005) NELIN, a new F-actin associated protein, stimulates HeLa cell migration and adhesion. Biochem Biophys Res Commun. **330:** 1127–1131.

Wang X, Osinska H, Klevitsky R, Gerdes AM, Nieman M, Lorenz J, Hewett T, and Robbins J. (2001) Expression of R120G-alphaB-crystallin causes aberrant desmin and alphaB-crystallin aggregation and cardiomyopathy in mice. Circ Res. **89:** 84–91.

Ward CL, Omura S, and Kopito RR. (1995) Degradation of CFTR by the ubiquitin-proteasome pathway. Cell. **83:** 121-127.

Wawrzynów A, and Zylicz M. (1995) Divergent effects of ATP on the binding of the DnaK and DnaJ chaperones to each other, or to their various native and denatured protein substrates. J Biol Chem. **270:** 19300-19306.

Wegele H, Müller L, and Buchner J. (2004) Hsp70 and Hsp90--a relay team for protein folding. Rev Physiol Biochem Pharmacol. **151:** 1-44.

Weintraub H, Tapscott SJ, Davis RL, Thayer MJ, Adam MA, Lassar AB, and Miller AD. (1989) Activation of muscle-specific genes in pigment, nerve, fat, liver, and fibroblast cell lines by forced expression of MyoD. Proc Natl Acad Sci. **86:** 5434–5438.

Weintraub H. (1993) The MyoD family and myogenesis: redundancy, networks, and thresholds. Cell. **75:** 1241–1244.

Welch WJ, and Suhan JP. (1985) Morphological study of the mammalian stress response: characterization of changes in cytoplasmic organelles, cytoskeleton, and nucleoli, and appearance of intranuclear actin filaments in rat fibroblasts after heat-shock treatment. J Cell Biol. **101:** 1198–1211.

Welihinda AA, Tirasophon W, and Kaufman RJ. (1999) The cellular response to protein misfolding in the endoplasmic reticulum. Gene Expr. **7:** 293-300.

Welsh MJ, and Gaestel M. (1998) Small heat-shock protein family: function in health and disease. Ann N Y Acad Sci. **851:** 28-35.

Wendler P, Shorter J, Plisson C, Cashikar AG, Lindquist SL, and Saibil HR. (2007) Atypical AAA+ subunit packing creates an expanded cavity for disaggregation by the protein-remodeling factor Hsp104. Cell. **131:**1366-1377.

Westwood JT, Clos J, and Wu C. (1991). Stress-induced oligomerlzatlon and chromosomal relocalization of heat-shock factor. Nature. **353:** 822-827.

Wickner S, Gottesman S, Skowyra D, Hoskins J, McKenney K, and Maurizi MR. (1994) A molecular chaperone, ClpA, functions like DnaK and DnaJ. Proc Natl Acad Sci **91:** 12218–12222

Wickner S, Maurizi MR, and Gottesman S. (1999) Posttranslational quality control: folding, refolding, and degrading proteins. Science. **286:** 1888-1893.

Wieske M, Benndorf R, Behlke J, Dölling R, Grelle G, Bielka H, and Lutsch G. (2001) Defined sequence segments of the small heat shock proteins HSP25 and alphaB-crystallin inhibit actin polymerization. Eur J Biochem. **268:** 2083–2090.

Willison K, Kelly A, Dudley K, Goodfellow P, Spurr N, Groves V, Gorman P, Sheer D, and Trowsdale J. (1987) Thehuman homolog of the mouse t-complex gene, TCP1, is located on human chromosome 6 but is not near the HILA region. EMBO J. **6:** 1967-1974.

Wistow GJ, and Piatigorsky J. (1988) Lens crystallins: the evolution and expression of proteins for a highly specialized tissue. Annu Rev Biohem. **57:** 479-504.

Wojtkowiak D, Georgopoulos C, and Zylicz M. (1993) Isolation and characterization of ClpX, a new ATP-dependent specificity component of the Clp protease of Escherichia coli. J Biol Chem **268:** 22609–22617

Wolynes PG, Onuchic JN, and Thirumalai D. (1995) Navigating the folding routes. Science. **267:** 1619-1620

Wu C, Clos J, Giorgi G, Haroun RI, Kim SJ, Rabindran SK, Westwood JT, Wisniewski J, and Yim G. (1994). Structure and regulation of heat shock transcription factor. In *The biology of heat shock proteins and molecular chaperones* 395–416. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Wu C. (1980) The 5' ends of Drosophila heat shock genes in chromatin are hypersensitive to DNase I. Nature. **286:** 854–860.

Wu C. (1984) Two protein-binding sites in chromatin implicated in the activation of heat-shock genes. Nature. **309:** 229–234.

Wu C. (1995). Heat shock transcription factors: structure and regulation. Annu. Rev. Cell Dev. Biol. **11:** 441–469.

Wu Z, Woodring PJ, Bhakta KS, Tamura K, Wen F, Feramisco JR, Karin M, Wang JY, and Puri PL. (2000) p38 and extracellular signal-regulated kinases regulate the myogenic program at multiple steps. Mol Cell Biol. **20:** 3951-3964.

Yablonka-Reuveni Z, Rudnicki MA, Rivera AJ, Primig M, Anderson JE, and Natanson P. (1999) The transition from proliferation to differentiation is delayed in satellite cells from mice lacking MyoD. Dev Biol. **210:** 440-455.

Yost HJ, Petersen RB, and Lindquist S. (1990) RNA metabolism: strategies for regulation in the heat shock response. Trends Genet. **6:**223-227.

Young JC, Moarefi I, and Hartl FU. (2001) Hsp90: a specialized but essential protein-folding tool. J Cell Biol. **154:** 267-273.

Zechner D, Thuerauf DJ, Hanford DS, McDonough PM, and Glembotski CC. (1997) A role for the p38 mitogen-activated protein kinase pathway in myocardial cell growth, sarcomeric organization, and cardiac-specific gene expression. J Cell Biol. **139:** 115-127.

Zhu X, Zhao X, Burkholder WF, Gragerov A, Ogata CM, Gottesman ME, and Hendrickson WA. (1996) Structural analysis of substrate binding by the molecular chaperone DnaK. Science. **272:** 1606-1614.

Zietkiewicz S, Krzewska J, and Liberek K. (2004) Successive & synergistic action of the Hsp70 and Hsp100 chaperones in protein disaggregation. J Biol Chem. **279:** 44376–44383

Zietkiewicz S, Lewandowska A, Stocki P, and Liberek K (2006) Hsp70 chaperone machine remodels protein aggregates at the initial step of Hsp70–Hsp100-dependent disaggregation. J Biol Chem. **281:** 7022–7029

Zu YL, Ai Y, Gilchrist A, Maulik N, Watras J, Sha'afi RI, Das DK, and Huang CK. (1997) High expression and activation of MAP kinase-activated protein kinase 2 in cardiac muscle cells. J Mol Cell Cardiol. **29:** 2159-2168.

List of abbreviations

bHLH	*basic helix loop helix*
BSA	*Bovine serum albumin*
Cdk	*Cyclin dependent kinase*
DM	*Differentiation medium*
DMEM	*Dulbecco's modified Eagle's medium*
DMSO	*Dimethyl sulphoxide*
EDTA	*Ethylenediamine tetra-acetic acid*
FACS	*Fluorescence activated cell sorter*
FBS	*Fetal bovine serum*
GM	*Growth medium*
$G_0/G_1/G_2$	*Gap phases 0, 1 and 2*
HEPES	*N-2-hydroxyethylpiperazine-N'-2-ethane sulphonicacid*
h	*hours*
IgG	*Immunoglobulin G*
IP	*Immuno precipitation*
kb	*kilobases*
kDa	*Kilo Dalton*
mA	*milli Amperes*
min	*minutes*
mM	*milli molar*
MRFs	*Muscle regulatory factors*
neo^R	*Neomycin resistant*
PAGE	*Polyacrylamide gel electrophoresis*
PBS	*Phosphate buffered saline*
PBST	*Phosphate buffered saline-Tween Triton X 100*
PCR	*Polymerase chain reaction*
pRb	*Retinoblastoma susceptibility gene product*
RNAi	*RNA interference*
rpm	*Revolutions per minute*
SDS	*Sodium dodecyl sulphate*
siRNA	*Small interfering RNA*
TE	*Tris-EDTA*
µg	*Microgram*
µl	*Micro liter*
µM	*Micro molar*