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Review

Small heat shock proteins: Role in cellular functions and pathology

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ABSTRACT

Small heat shock proteins (sHsps) are conserved across species and are important in stress tolerance. Many sHsps exhibit chaperone-like activity in preventing aggregation of target proteins, keeping them in a folding–competent state and refolding them by themselves or in concert with other ATP-dependent chaperones. Mutations in human sHsps result in myopathies, neuropathies and cataract. Their expression is modulated in diseases such as Alzheimer's, Parkinson's and cancer. Their ability to bind Cu^{2+}, and suppress generation of reactive oxygen species (ROS) may have implications in Cu^{2+}-homeostasis and neurodegenerative diseases. Circulating αB-crystallin and Hsp27 in the plasma may exhibit immunomodulatory and anti-inflammatory functions. αB-crystallin and Hsp20 exhitbit anti-platelet aggregation: these beneficial effects indicate their use as potential therapeutic agents. sHsps have roles in differentiation, proteasomal degradation, autophagy and development. sHsps exhibit a robust anti-apoptotic property, involving several stages of mitochondrial-mediated, extrinsic apoptotic as well as pro-survival pathways. Dynamic N- and C-termini and oligomeric assemblies of αB-crystallin and Hsp27 are important factors for their functions. We propose a "dynamic partitioning hypothesis" for the promiscuous interactions and pleotropic functions exhibited by sHsps. Stress tolerance and anti-apoptotic properties of sHsps have both beneficial and deleterious consequences in human health and diseases. Conditional and targeted modulation of their expression and/or activity could be used as strategies in treating several human disorders. The review attempts to provide a critical overview of sHsps and their divergent roles in cellular processes particularly in the context of human health and disease.

1. Introduction

Organisms and cells respond to various stress conditions such as environmental, metabolic or pathophysiological stress by selectively up-regulating the expression of a group of proteins called the heat shock proteins (Hsps). Based on their molecular masses these proteins have been classified into six major families i.e. Hsp100, Hsp90, Hsp70, Hsp60, Hsp40 and small heat shock proteins (sHsps). sHsps have subunit molecular masses of 12-43 kDa and are characterized by the presence of a highly conserved stretch of 80-100 amino acids in their C-terminal domains called the "α-crystallin domain" (ACD) that is flanked by less conserved (except a few stretches) N-terminal domain (NTD) and C-terminal extension (CTE) [1–3]. Some members of the sHsp family such as Hsp27, αA- and αB-crystallin form large oligomeric species.

This review essentially deals with mammalian sHsps. Ten sHsps (named HspB1 to HspB10) have been identified in the human genome based on sequence homology [1]. For easy identification we have used the old nomenclature for HspB1 (Hsp27), HspB4 (αA-crystallin), HspB5 (αB-crystallin), HspB6 (Hsp20) and HspB8 (Hsp22). They have been categorized as Class I or Class II [4]. Hsp27, αB-crystallin, Hsp20 and Hsp22 are Class I sHsps (Table 1). They are distributed widely, are found in various tissues, are predominantly heat-inducible (Hsp22 heat-inducibility is cell type dependent [5]) and play an important role in cell survival under conditions of stress. Class II sHsps include HspB2, HspB3, αA-crystallin, HspB7, HspB9 and HspB10 which exhibit a tissue-restricted pattern of expression (Table 1). Class II sHsps are believed to play a major role in development, differentiation and specialized tissue-specific functions [4].

Besides the molecular chaperone-like activity in preventing aggregation of proteins/peptides, sHsps such as Hsp27 and αB-crystallin, are involved in diverse cellular functions such as stress tolerance, protein folding, protein degradation, maintaining cytoskeletal integrity, cell death, differentiation, cell cycle and signal transduction and development [6–12]. Members of the sHsp family exhibit cardio and neuroprotection, potent anti-apoptotic activity, pro-angiogenic property and anti-inflammatory property involving interactions with several clients. The promiscuous interactions and pleotropic functions of sHsps and the underlying molecular mechanism is not completely understood. These roles of sHsps have important implications in general health and disease conditions. Hsp27 and αB-crystallin have been proposed to be therapeutic targets [see review 13].

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Table 1
Nomenclature, distribution and functions of human small heat shock proteins (see review [4] and references therein for details).

Name	Subunit Mol. Mass (kDa)	pI	Tissue distribution	Stress-inducibility	Class	Functions
Hsp27 (HspB1)	22.8	6.4	Ubiquitous, high levels in heart, striated and smooth muscles	+	I	Chaperone activity, stabilization of cytoskeleton, anti-apoptotic and anti-oxidant function
HspB2 (MKBP)	20.2	4.8	Heart, skeletal and smooth muscle	-	II	Chaperones DMPK and enhances its kinase activity. Target protein-dependent chaperone activity, myofibrillar integrity, anti-apoptotic function, mitochondrial energetic, anti-apoptotic
HspB3	17.0	5.9	Heart, brain, skeletal and smooth muscle	-	II	Target protein-dependent chaperone activity, Maintaining myofibrillar integrity
αA-crystallin (HspB4)	19.9	6.2	Abundant in eye lens. skeletal muscle, liver, spleen, adipose tissue (low level)	-	II	Chaperone activity, genomic stability, eye lens refractive index
αB-crystallin (HspB5)	20.2	7.4	Ubiquitous, abundant in eye lens. High levels in heart and muscle	+	I	Chaperone activity, stabilization of cytoskeletal and nucleoskeletal matrix, cell cycle, cardioprotection, eye lens refractive index, regulation of muscle differentiation, anti-apoptotic function
Hsp20 (HspB6)	16.8	6.4	Ubiquitous, abundant in muscle	-	I	Smooth muscle relaxation, cardioprotection, chaperone activity, anti-apoptotic
HspB7(cardiovascular heat shock protein)	18.6	6.5	Heart and skeletal muscle. Adipose tissue (low level)	-	II	Chaperone activity, maintaining myofibrillar integrity,
Hsp22 (HspB8, E21G1, α-crystallin C)	21.6	4.7	Ubiquitous	+ (cell type dependent)	I	Chaperone activity, induction of autophagy
HspB9 (Hest shock protein beta-9, cancer/testis antigen 51 (CT51))	17.5	9.0	Testis	-	II	Role in cancer/testis antigen
HspB10 (Outer dense fibre of sperm tails, ODF27, ODFPG, RT7, ODFP, CT133)	28.3	8.4	Testis	-	II	Elastic cytoskeletal structure

This review highlights the important findings on the chaperone activity, structural aspects and the role of sHsps in various physiological and pathophysiological processes. In one of the following sections, we propose a "dynamic partitioning hypothesis" for promiscuous interactions and pleotropic functions of sHsps. The general and potent protective functions of some of the sHsps, particularly Hsp27, αB-crystallin and Hsp22, seem to be exploited by cancer cells for their survival, proliferation and metastasis. It emerges that developing strategies for conditional and targeted modulation of expression and functions of sHsps is important for management of several disease conditions. Some sHsps are found extracellularly and exhibit immunomodulation and anti-platelet aggregation properties, implying that these sHsps and some of their derived peptides may serve as potential therapeutic molecules.

1.1. Chaperone-like activity of small heat shock proteins

The ability to prevent the aggregation of proteins and polypeptides is the most important function of many sHsps, especially under conditions of stress that lead to unfolding of cellular proteins. This chaperone-like function was first shown for the eye lens protein, α-crystallin, a heterooligomer of two gene products, αA- and αB-crystallin, against the heat-induced aggregation of β-crystallin, γ-crystallin and alcohol dehydrogenase [14]. Murine Hsp25, human Hsp27 and bovine αB-crystallin prevent aggregation of citrate synthase and α-glucosidase [15]. Studies from our laboratory showed that α-crystallin prevented the photo-aggregation of γ-crystallin [16,17], thermal aggregation of ζ-crystallin, and carbonic anhydrase [18,19], DTT-induced aggregation of α-lactalbumin [20] and refolding-induced aggregation of β-crystallin [21]. Homo-oligomers of αA- and αB-crystallin also exhibit chaperone property in preventing aggregation of various target proteins [22–25].

Human Hsp20 forms dimers [26,27] and exhibits chaperone-like activity in preventing the DTT-induced aggregation of insulin and the heat-induced aggregation of yeast alcohol dehydrogenase [26]. Rat Hsp20 forms 43-kDa dimers and 470-kDa multimers with the relative ratio depending on protein concentration and exhibits poor chaperone-like activity [28]. We have earlier shown that the heat-induced expression of Hsp22 is cell type-dependent [5]. Studies from our laboratory [5] and those of others [29] have shown that Hsp22 prevents aggregation of several target proteins.

Studies from our laboratory have shown that HspB2 effectively prevented the aggregation of alcohol dehydrogenase and insulin but not that of citrate synthase [30]. HspB3, prevented the aggregation of alcohol dehydrogenase very effectively, and that of citrate synthase at higher chaperone to target protein ratios but did not prevent the aggregation of insulin [31]. Thus, unlike Hsp27, α-crystallins and Hsp22, which are general chaperones, the chaperoning ability of HspB2 and HspB3 appears to be target protein-dependent [30,31].

sHsps prevent the aggregation of target proteins by forming complexes with the aggregation-prone partially unfolded states of the target proteins [19,20,25,32]. We have earlier shown two types of interactions of αA- and αB-crystallin with thermally induced unfolding intermediates of citrate synthase – a reversible relatively weak interaction with early unfolding intermediates that helps in refolding the enzyme to its active state, and an irreversible interaction with aggregation-prone late unfolding intermediate keeping it in soluble complex [25]. Two modes of interactions, namely low affinity and high affinity modes, of αA- and αB-crystallin and Hsp27 with destabilized β-crystallin, T4 lysozyme and its destabilized mutants, have also been reported by another laboratory [33–36]. The target proteins bound to sHsps *in vivo* could be either subjected to refolding or to degradation as discussed later.

1.2. Temperature-dependence of chaperone-like activity

α-Crystallins, Hsp27, Hsp22 and HspB2 exhibit temperature-dependent chaperone property in preventing aggregation of target proteins. Studies from our laboratory have shown the temperature-dependent chaperone-like activity of α-crystallin using various aggregation systems [16–18,21,37]. Temperature-dependent conformational change in α-crystallin leading to the increased exposure of hydrophobic surfaces paralleled the increase in chaperone-like activity; α-crystallin interacted with partially unfolded proteins through appropriately placed hydrophobic surfaces and prevented their aggregation [17,18,21,37]. Homo-oligomers of αA- and αB-crystallin also exhibit temperature-dependent chaperone-like activity [22,23]. Hsp27 undergoes thermally induced self-association, leading to increased oligomeric size, which correlated with increase in its chaperone-like activity [38]. Rat Hsp22 exhibits heat-induced conformational changes with increased exposure of hydrophobic surfaces and chaperone-like activity [5]. HspB2 acquires a molten globule-like state and exhibits a monotonous increase in its chaperone-like activity at elevated temperatures [30].

Hydrogen-deuterium exchange of amide proton of α-crystallins by NMR spectroscopy showed that hydrophobic regions around the residues 32-37 and 72-75 of αA- and 28-34 of αB-crystallin were exposed above 30 °C [39]. More chaperone sites for target proteins are accessible at higher temperatures (~60 °C) of α-crystallin complex saturated with a different target protein at lower temperature (37 °C) [40]. Studies also demonstrated an increased rate of subunit exchange for α-crystallins [41,42] and Hsp27 [43], resulting in higher chaperone-like activity at higher temperatures relevant to physiological heat-stress. However, glutaraldehyde-cross linked bovine eye lens α-crystallin exhibited higher chaperone-like activity than the native protein, indicating that dissociation of subunits and chaperone-like activity are not correlated [44]. A mutant (S4C) yeast Hsp26 which cannot dissociate to dimers and exhibits significantly reduced subunit exchange shows chaperone-like activity comparable to that of the native Hsp26 upon thermal activation [45]. Therefore, in addition to dynamic subunit exchange, factors such as tertiary structural perturbation may be involved upon thermal activation of sHsps. It appears that sHsps exist as populations of states at equilibrium controlled by temperature. The population at lower temperature is relatively less efficient, while temperature-induced redistribution of populations having altered conformation (for example, tertiary structural perturbation [21]) with appropriately placed, probably contiguous hydrophobic surfaces exhibit potent chaperone activity.

1.3. Effect of small molecules on the chaperone-like activity of sHsps

Studies have shown that perturbing the structure of sHsps such as lens α-crystallin or homooligomers of αA- and αB-crystallin as well as Hsp27 can increase the chaperone-like activity. Exposure of α-crystallin to low concentrations of urea [17] or guanidine hydrochloride [46] has been shown to perturb its structure, resulting in increased chaperone activity.

As loss or decreased chaperone activity may result in disease susceptibility, the ability to enhance the chaperone-like activity using biocompatible small molecules could be of therapeutic importance. Small biocompatible molecules such as ATP [47], pantethine [48], arginine hydrochloride and aminoguanidine hydrochloride [49–51] have also been shown to increase the chaperone-like activity of α-crystallin or that of the homo-oligomers of αA- and αB-crystallin. Exposure to arginine hydrochloride and aminoguanidine hydrochloride resulted in subtle changes in tertiary structure, significant changes in the quaternary structure, increased exposure of hydrophobic surfaces [49] and enhanced rate of subunit exchange [50] of α-crystallin as well as αA- and αB-crystallin homooligomers. It is possible that arginine hydrochloride and aminoguanidine hydrochloride also affect the structure of target proteins. However, since under the experimental conditions (destabilizing), the model proteins continued to aggregate even in the presence of these compounds, the observed increase in the suppression of aggregation by sHsp can be attributed to increased chaperone activity due to the observed structural and dynamic alteration of sHsp by these compounds. Interestingly, arginine hydrochloride could also enhance the chaperone-like activity of the R120G mutant of αB-crystallin, which is associated with congenital cataract and desmin-related myopathy [51]. Methylglyoxal modification increased the chaperone-like activity of Hsp27 towards the heat-induced aggregation of citrate synthase [52].

α-Crystallins and Hsp27 bind ATP (but do not hydrolyse) in their ACDs [47,53–56]. ATP has been shown to bind in the groove of β4-β8 strands of the ACD of αB-crystallin [57] (structural aspects are discussed in detail later). ATP-binding leads to increased chaperone activity of αB-crystallin [53]. Interestingly, the β4-β8 groove has also been shown to interact with CTE [58–60] and these regions are also implicated in interactions with certain substrates [61]. It would be interesting to find out whether ATP-binding would compete or allosterically control interaction of the CTE and/or substrate binding to αB-crystallin.

1.4. Reactivation of enzymes

The fate of the target proteins of sHsps can be (i) reactivation to their active state or (ii) degradation either by proteosomal or autophagy pathways. sHsps have been demonstrated to reactivate target proteins through at least three different mechanisms which are described below.

Hsp27 and α-crystallins interfere in the kinetic partitioning between productive folding to active state and aggregation of target proteins [15,25]. Interaction of α-crystallins with early unfolding intermediates of citrate synthase reduces their partitioning into aggregation-prone intermediates and results in enhanced population of early unfolding intermediates that can be refolded to active state [25]. However, α-crystallins form a soluble, stable complex with aggregation-prone late unfolding intermediates of citrate synthase [25]. Murine Hsp25, human Hsp27 or bovine αB-crystallin have been shown to increase the refolding yields of citrate synthase and α-glucosidase upon refolding from their urea-denatured state [15]. Presence of αA- and αB-crystallin leads to increased refolding yield of the tetrameric quinone oxidoreductase (ζ-crystallin) from the urea-denatured partially unfolded state with molten-globule like characteristics [62]. αB-crystallin improves the reactivation of glucose-6-phosphate dehydrogenase (G6PD) upon refolding from its completely denatured state [63]. Thus, the chaperone activity of α-crystallins involves both transient/reversible and stable interactions depending on the nature of intermediates on the unfolding pathway; one leads to reactivation of the enzyme activity while the other prevents aggregation [25,62].

Another mechanism by which sHsps reactivate target proteins involves binding of ATP leading to conformational changes of the complex and release of the refolding-competent target proteins. ATP-binding to αB-crystallin not only increases the suppression of heat-induced aggregation of target proteins, but also significantly enhances reactivation yield [55]. α-Crystallin has been shown to bind to the molten globule states of protein such as HIV-1 protease, xylose reductase and xylanase II, which can be refolded in the presence of ATP [64–66].

The concerted action of sHsps (termed as "holdases' [67]) in keeping folding-competent targets in solution, and of other ATP-dependent chaperones in reactivation of enzymes has also been demonstrated. sHsps together with molecular chaperones from the Hsp70 and Hsp100 family serve to maintain "proteostasis" in cells [see review 68]. Treating the α-crystallin-target protein complex with either rabbit reticulocyte lysate (as a source of chaperones) or with purified Hsp70, Hsp40, Hsp60 and an ATP-generating system, led to the reactivation of the target proteins (luciferase and citrate synthase) [69]. Binding of ATP to sHsps caused a conformational change in the sHsp, leading to the release of the target protein, which was then reactivated in the presence of Hsp70 [70]. Unfolded intermediate(s) of citrate synthase bound

by Hsp25 has been shown to be refolded in the presence of Hsp70 and ATP [71]. αB-crystallin-bound malate dehydrogenase can be refolded to its active state by the Hsc70 chaperone machinery [72]. This concerted mechanism has also been demonstrated in prokaryotic chaperone systems - *E.coli* sHsp IbpB bound and stabilized denatured proteins which were demonstrated to be folded to their active state by subsequent action of the DnaK/DnaJ/DnaE and the GroEL/GroES chaperone systems [73]. sHsps of *E.coli*, IbpA and IbpB function in cooperation with ClpB and DnaK system in cell survival and heat tolerance, forming a functional triade [74]. A study on the cooperative functions of chaperones in *S. cerevisiae* cytosol shows that during the reactivation process, substrate proteins are transferred from their complex with Hsp26 to Hsp104 and Hsp70 (Ssa1) [75]. Thus, sHsps form a part of the cellular chaperone network, preventing aggregation of target proteins and maintaining them in a folding-competent state, facilitating their release in an active state with the co-operation of ATP-dependent chaperones.

1.5. sHsps may chaperone membrane proteins

αB-crystallin binds and prevents the aggregation of the mutant form of two multispan transmembrane proteins and helps them attain the proper functional folded state [76]. It prevents the formation of inter-chain disulfide bridges between the lumenal ectodomains of the aggregated mutant chains of Frizzled4 (responsible for a rare autosomal dominant form of familial exudative vitreoretinopathy (Fz4-FEVR)), enabling correct folding and appropriate compartmentalization on the plasma membrane. αB-crystallin helps the H1069Q mutant of ATP7B Cu transporter that is associated with a common form of Wilson's disease to fold into a proper conformation, to move to the Golgi complex, and to respond to copper overload like wild-type ATP7B [76]. It appears that in the cases of mutants that fail to attain the functional state, sHsps can target them to degradation. For example, Hsp27 binds to the mutant F508del cystic fibrosis transmembrane conductance regulator (CFTR), and targets the mutant CFTR to proteasomal degradation through F508del SUMOylation [77]. Aquaporin 0 (AQP0) (also known as membrane intrinsic protein (MIP)) which is expressed exclusively in the lens during terminal differentiation of fiber cells plays an important role in regulation of water content and in cell-to-cell adhesion of the lens fiber cells. The eye lens α-crystallin and the homo-oligomers of αA- and αB-crystallin were able to prevent the heat-induced aggregation of AQP0 and stabilize its secondary structure from heat-induced unfolding by the formation of a stable complex [78].

sHsps bind to the aggregation-prone mutant membrane proteins and keep them as soluble complexes. The subsequent mechanistic events which determine the fate of the protein - namely, targeting it to the appropriate destination in the functional state or targeting it to degradation - are still to be understood. It is interesting that some of the sHsps have also been shown to associate with membranes. For example, both homo- and hetero-oligomers of αA- and αB-crystallin are known to interact with lipid vesicles [79]. Association of α-crystallin with membranes has been shown to modulate lipid membrane polymorphism: stabilizing liquid crystallin state of the bi-layer membrane [80]. A study from our laboratory has shown that Hsp22 exhibits membrane localization in cells and binds to lipid vesicles *in vitro* [81].Thus, we hypothesise that the inherent tendency of sHsps to associate with membrane would facilitate the sHsps-membrane protein (and/or their mutants) complex to be in proximity to the membrane surface, which would help in incorporation of the membrane proteins by preferential partitioning to the membrane and either concerted or subsequent folding in the membrane environment. Thus, there are preliminary indications of involvement of some sHsps in membrane protein quality control which need further studies both with respect to phenomenology and mechanism.

1.6. Mutations of sHsps lead to neuropathies and myopathies

The pathological conditions due to mutations or modifications in chaperones are generally termed as chaperonopathies [see review 82]. Detailed description of the various studies on the mutational effects on sHsps accumulated over the period would be very exhaustive and beyond the scope of the present focus of the review. The subject has been specifically reviewed elsewhere [83–86]. Mutations or modifications in sHsps can lead to adverse pathological conditions due to either (i) loss of their function leading to compromise in the ability of the cells to cope up with stress, or (ii) toxic gain of function due to misfolding and aggregation or improper interaction of the mutant chaperone molecules themselves or with other cellular targets. Chaperonopathies have been further classified as genetic or acquired, either by aging or due to post-translational modifications. Decreased expression of αA-crystallin with age and its post-translational changes in the retina resulting in retinopathy is an example of acquired chaperonopathy [87].

Several mutations in sHsps in human are associated with pathological conditions, mostly neuropathies and myopathies [see reviews 83–86]. Most of the mutations in sHsps are associated with similar but not identical disease conditions, indicating that the sHsps, in addition to their similar functions, also have distinct roles. Therefore, the exact mechanism(s) by which each mutation causes disease could be different, but converge into the pathways that are critical for survival and function of particularly, muscle and neuronal tissues. Many of the mutations are genetically dominant negative, implying that toxic gain of function could be a dominant contributing factor in the pathogenesis. For example, R120G mutation in αB-crystallin that leads to desmin-related myopathy and congenital cataract [88] alters oligomeric size and decreases chaperone activity [24,89]. R120GαB-crystallin forms inclusions in cells that have amyloid-like character and exhibit propensities to form amyloid-oligomers which are highly cytotoxic [90]. Further studies addressing the exact mechanism(s) by which individual mutations manifest pathology and evolving strategy to tackle the mutation-induced adverse effect are important to develop therapeutic approaches for these chaperonopathies. The observation that overexpression of other sHsps could rescue cells from, for example, R120G αB-crystallin induced formation of intracellular inclusions and cell death [91–93], provides a potential strategy to alleviate the toxic effect of the mutant protein(s). A peptide aptamer has been shown to abolish the dominant negative effect of R120G αB-crystallin by disrupting its interaction with Hsp27 [94]. Inhibition of histone deacetylase 6 in R120GαB-crystallin transgenic mice leads to hyperacetylation of tubulin and decreased aggregation of proteins and improved cardiac function [95]. Therefore, prevention of aggregate formation of R120GαB-crystallin and/or its aberrant interactions with other proteins would be potential modalities for treatment.

1.7. Structural aspects of sHsps

As mentioned earlier, sequence alignment of the sHsps shows a highly conserved ACD which is flanked by the NTD and CTE, both of which are variable in length and sequence except for a few conserved stretches [1–3,96]. Crystal structures of the full-length prokaryotic sHsp, *Methanococcus jannaschii* sHsp16.5 [97] and the plant sHsp, wheat sHsp16.9 [98] that form mono-dispersed discrete oligomers are available. Mammalian sHsps such as Hsp27, αB-crystallin, in general, exist in polydispersed oligomeric populations and crystal structures of full-length proteins have not yet been determined. However, crystal structures of the ACDs of Hsp27, αA-crystallin, αB-crystallin and Hsp20 show β-sheet rich immunoglobulin-like fold [99–103]. Fig. 1 shows dimeric arrangement of ACD of αB-crystallin and the models proposed for a 24-mer oligomeric assembly of full-length αB-crystallin. The ACD is primarily involved in inter-subunit interaction [100,101] leading to dimerization. Both crystallography and solid state NMR studies have shown that the dimeric 'building block' is composed

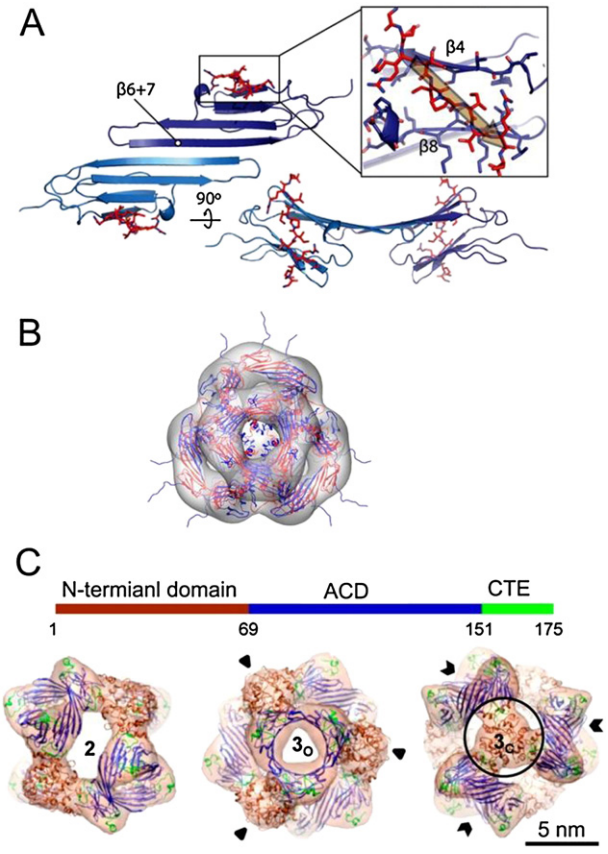

Fig. 1. Structural aspects of αB-crystallin. (A) Crystal structure of dimeric ACD of αB-crystallin along with the peptide with the palindromic sequence, ERTIPITRE, derived from the C-terminal extension (CTE) of the protein (shown red) bound to each monomer [103]. The dimer interface is formed by pairwise and antiparallel interactions between extended β6 + 7 strands from each monomer [103]. Inset highlights the palindromic peptide (yellow arrow shows the N-to-C direction) containing the IXI motif bound to the hydrophobic groove between β4-β8 strands in an antiparallel direction to the β8 strand [103]. (B) Oligomeric (24-mer) model of αB-crystallin superimposed on the electron densities of EM of αB-crystallin proposed by Jehle et al. [106]. Three dimers (two monomers colored differently) form a triangular array (hexamer) on the surface of a multimer, each connected via its IXI motif bound in the β4-β8 groove of a neighboring dimer [106]. Four triangular hexamers arranged with tetrahedral symmetry (shown by the negative stained EM of αB-crystallin [105]) results in four threefold axes and three twofold axes of symmetry with a central cavity (~4 nm dia). Flexible residues of the N- and C-termini identified by NMR were modeled based on different orientations of the ACDs, and the IXI motifs bound to them: half the subunits in a 24-mer have their C-terminal residues 166–175 on the surface and half have them pointing to the interior [106]. N-terminal residues 1–10 were loosely packed in the interior of the model [106] that fits the radius of gyration measured by SAXS [106] and the EM data of Peschek et al. [105]. (C) Pseudoatomic model of a 24-meric αB-crystallin assembly proposed by Braun et al. [111] based on 3D-constructed cryo EM and molecular modeling taking support from the results of NMR spectroscopy, chemical cross-linking, mass spectrometry and previous negative stained EM of αB-crystallin [105]. Different views of the oligomer with the docked model of αB-crystallin 24-mer superimposed on cryo-EM density map - viewed along a two- (Left) and a threefold symmetry axis intercepting the area (3o)(Center), and area showing α-helical segments of the N-terminal domain (3c) (Right). The flexible C-termini appear to face outward (surface) as well as inward (towards inner cavity) in the oligomeric (24-mer) assembly of αB-crystallin in both the models (B & C). However, the models seem to differ in the spatial disposition of the flexible N-terminal regions – the region is arranged inward to the central cavity in (B) while it is arranged towards the surface in (C). Fig. 1(A-C) are taken from references [103,106,111] respectively with permission from PNAS, USA.

of a β-sandwich ACD core that assembles through anti-parallel pairwise interactions between extended β6-β7 strands [58,59,100,102,103]. This dimer interface seems to be common to metazoan sHSPs, and was found to occur in different registration states [58,59,100,102,103], termed API, APII and APIII [60,103]. In solution, the ACD of αB-crystallin predominantly exists with a dimeric intereface of APII register [103]. The dimer serves as a building block for higher order oligomerization in sHsps [69,100–104]. Most of the structural information on oligomeric assembly available is with respect to αB-crystallin. Clubbing information from biophysical data on the oligomeric size and image reconstruction using negative stained electron micrograph of αB-crystallin, an oligomeric structure of 24-mer with a central cavity has been proposed [105]. Based on solid state NMR studies and small-angle X-ray scattering (SAXS) studies, an oligomeric structure of 24-mer has been proposed [58].

A model of full-length αB-crystallin as a symmetric multimer of 24 subunits involving a hierarchy of interactions of the ACD, the CTE and the NTD has been proposed based on solid-state NMR, SAXS [106], and electron microscopy (EM) data [105]: Interactions between two ACDs define a dimer. Three dimers, connected by the conserved IXI motif from the C-terminus of one dimer binding to hydrophobic pockets formed by the β4 and β8 strands on the edge of another dimer, define a hexameric unit. Variable interactions (and hence multiple environments contributing to the heterogeneity in NMR signals) involving the NTD form higher-order multimers [106]. Truncation of the NTD [106–108] or the deletion of conserved stretches, SRLFDQFFG [109] and FLRAPSWF [110] leads to formation of dimer to hexamer or smaller oligomers of αB-crystallin, consistent with their role in building higher order oligomeric structure. Further association of dimeric building blocks on to existing openings in the shell of the 24-mer give rise to higher-order multimeric assembly such as 26-, 28-mers, as the EM density contains six gaps with dimensions that can accommodate a dimer at the edges of the three two-fold axes of the 24-meric species [106].

Another study has also proposed models of polydispersed species of αB-crystallin ranging from 12-mer to 48-mer including the odd numbered multimeric species of 23-mer [111]. αB-crystallin oligomers showed size and structural variability on cryo-electron micrographs and about 30% of the particles could be assigned to the 24-meric assembly with a 3D reconstructed model at 9.4-Å resolution [111]. The αB-crystallin 24-mer is a hollow, spherical complex of approximately 13.5-nm diameter in which subunits are arranged according to tetrahedral symmetry [111]. Two types of dimers have been proposed while building the oligomeric assembly by molecular modeling using constraints from NMR data [58], the density maps of cryo-electron micrographs, chemical cross-linking and mass spectrometry: an elongated dimer, where the N- and C-termini come in close proximity, and a bent dimer, where both the termini are disposed to opposite ends, are arranged in a hexameric unit. The hexameric units in multiples or with added monomer or dimeric units can be further assembled into several multimeric species with reconstructed images represented in the cryo-electron micrographs [111]. The model also shows that the phosphorylation sites (S19, S45, S59) at the NTD are in close proximity in the hexamer unit due to the assembly of N-terminals from three dimeric units, suggesting that charge repulsion due to phosphorylation can destabilize the oligomeric structure [111]. Cryo EM shows shifting of mean populations of αB-crystallin to hexamer and 12-mer in phosphorylation-mimicking mutants, while retaining the structural features of the hexameric assembly of the wild type protein [72].

The flexible C-termini appear to face outward (surface) as well as inward (towards inner cavity) in the oligomeric (24-mer) assembly of αB-crystallin in both the models (Fig. 1B and C see [106,111] for details). However, the models seem to differ with respect to the spatial disposition of the flexible N-terminal regions – the region is arranged inward to the central cavity in the model proposed in [106] (Fig. 1B), while it is arranged towards the surface in the model proposed in [111] (Fig. 1C). The phosphorylation sites (S19, S45, S59) of αB-crystallin (discussed later) would be readily accessible to the kinases if the NTDs are disposed on the surface of the assembly, whereas subunit dissociation would be the rate-limiting factor for phosphorylation events if the NTDs are disposed inside the assembly. Whether phosphorylation of αB-crystallin takes place in the oligomeric state or in the disassociated states that are in equilibrium with the oligomeric assembly needs to be investigated. Though the proposed models have given considerable insights into the structural aspects of αB-crystallin, we are still far from giving an

adequate structural basis, especially for the pleotropic functions of the protein as well as those of sHsps in general. Considering the plasticity of assembly as well as the dynamic (highly flexible) nature of the N- and C-terminal regions [106,112], sHsps may exist in a wide spectrum of interconverting conformational ensembles. The proposed models of αB-crystallin [58,105,106,111] might represent a few of these ensembles. The temperature-dependent faster subunit exchange process appears to be a unique feature of polydispersed vertebrate sHsps as well as monodispersed plant sHsps (rate constants in range of min^{-1}) as opposed to very slow subunit exchange rate of other multimeric proteins (rate constants in the range of day^{-1}) such as tetrameric transthyretin or hepatitis B capsid protein [see review 112]. Therefore, further understanding on structural and dynamic aspects with respect to other sHsps is important. Structural information on individual sHsps would also provide information on the hetero-sHsp interactions - interface between hetero-subunits might create new target interaction sites.

1.8. Structural-chaperone functional relationship of sHsps

Interchanging either the NTDs [113] or the CTEs [114] between αA- and αB-crystallin resulted in chimeric proteins which were very different in oligomeric size and chaperone-like activity from their wildtype counterparts. Fusing the CTE of αB-crystallin to HspB3, which lacks a CTE, modulated its chaperone-like activity and its oligomerization [31]. Thus, oligomeric assembly and chaperone activity of a given sHsp is inter-dependent on its NTD, ACD and CTE.

The NTD has a preponderance of hydrophobic residues and provides sites for interactions leading to higher order oligomerization of sHsps. Truncation of sequences from the NTD of α-crystallin leads to formation of tetrameric species with decreased chaperone activity and loss of subunit exchange with other sHsps [108,115–117]. The conserved sequence, (e.g., SRLFDQFFG of the NTD of α-crystallins) has a propensity to adopt an amphipathic helix, and deletion of this sequence leads to a significant decrease in oligomeric size and increased chaperone activity, indicating that this region contributes to the higher order oligomerization of αA- and αB-crystallin [109]. Similarly, deletion of ^{54}FLRAPSWF^{61} also results in decreased oligomeric size, increased accessible hydrophobic surface and enhanced chaperone activity [110].

The individual regions, the NTD, ACD and the CTE of α-crystallins display marked differences in their biophysical properties but each of them independently exhibits chaperone activity to varying extents, the N-terminal domain being the most active [118]. The NTD of Hsp27 is dynamic and binds to the destabilized target protein, T4 lysozyme [119]. Studies using a cleavable fluorescent, photoactive cross-linking agent sulfosuccinimidyl-2 (7-azido-4-methylcoumarin-3-acetamido)-ethyl-1,3' dithiopropionate (SAED) and mass spectroscopic analysis showed that the regions that ^{57}APSWIDTGLSEMR^{69} and ^{93}VLGDVIEVHGKHEER^{107} in αB-crystallin were involved in binding to heat-denatured yeast alcohol dehydrogenase, whereas regions ^{75}FSVNLDVK^{82} of αB-crystallin and ^{12}RTLGPFYPSR^{21} and ^{71}FVIFLDVKHFSPEDLTVK^{83} of αA-crystallin were identified as melittin-binding sites in α-crystallin [120,122]. Using peptide array approach, it has been shown that many peptides derived from the NTD, ACD and the CTE of αB-crystallin interacted with regulatory proteins such as basic fibroblast growth factor (FGF-2), nerve growth factor (NGF), vascular endothelial growth factor (VEGF), insulin and β-catenin), which also partly overlapped with sequences that interact with partially unfolded proteins, suggesting a common function for αB-crystallin in chaperone activity and the regulation of cell growth and differentiation [61]. Mutational analysis of the β8 strand of αB-crystallin reveals involvement of this strand in the chaperone property of the protein [122]. Sequences corresponding to β strands 3, 8 and 9 from ACD of αB-crystallin seem to be the common sites for interactions with various substrates for chaperone activity and some regulatory proteins [61].

The CTE has a preponderance of charged residues and was believed to play a solubilizer role in keeping the sHsps and their target protein complexes in solution [123]. Our earlier study [124] showed that the conserved IXI/V motif [96] in the CTE of αB-crystallin is involved in inter-subunit interactions. Mutations of the isoleucine residues in the IXI/V motif and truncations in bacterial and plant α-sHsps result in dissociation of the subunits and loss of chaperone-like activity [125,126]. On the other hand, mutation of the hydrophobic residues of the motif to glycine leads to alternative oligomeric assembly and increased chaperone activity in αA- and αB-crystallin [124]. Subsequent solid state NMR and small angle X-ray scattering (SAXS) studies on αB-crystallin show that the IXI (I159-P160-I161) motif is involved in interdimeric and intermolecular interactions by binding to the substrate-binding groove (β4 and β8 strands), which is released upon decrease in pH, potentially leading to chaperone activation [59]. Crystal structures of the NTD-truncated αA-crystallin (residues 59–163), and αB-crystallin (residues 68–162) reveal bidirectional binding of the C-terminal tail sequence IXI/V in the groove of the core formed by strands β4 and β8 [59].

The palindromic sequence around the IXI/V motif in αA- (ERAIPVSRE) and αB-crystallin (ERTIPITRE) would allow bidirectional interactions at the subunit interface and the dimeric interface. An antiparallel β sheet with three possible registration shifts (AP1 to AP3) potentially enforces polydispersity of α-crystallins, which could be evolved as mechanisms for chaperone action and for prevention of crystallization, both necessary for transparency of eye lenses [59]. NMR relaxation experiments have revealed that the CTE of αB-crystallin is highly dynamic. Its inter-subunit interactions (via the IXI motif) are temperature-dependent and only a fraction of the molecule exhibits binding of the IXI motif at physiological temperature [127]. Such dynamic fast exchange of the CTE between bound and free states forms the regulatory mechanism in subunit exchange, polydispersity and chaperone activation. NMR studies showed that the IXI motif-containing peptide derived from the CTE of αB-crystallin, PERTIPITREEK, binds to the core ACD dimer of αB-crystallin and replacement of the isoleucine residues of the motif by alanine or glycine abolishes the binding [60]. Moreover, IXI-mimicking peptides derived from the CTEs of αA-crystallin, Hsp27 and HspB2 also show binding to the ACD of αB-crystallin [60].

A mass spectrometric study on binding of the palindromic peptide residues 156–164 (ERTIPITRE) or the peptide residues 156–175 (ERTIPITREEKPAVTAAPKK) of αB-crystallin to the dimers of ACD shows that these peptides bind weakly to the domain and binding of a second peptide to the dimer is less favoured [128]. The mass spectral data on distribution of oligomeric species and subunit exchange process of αB-crystallin were modeled as follows: (i) two interactions between individual αB-crystallin monomers, corresponding to intra-dimer and inter-dimer interfaces, (ii) individual oligomers are in dynamic equilibrium with their corresponding monomers (explaining the appearance of oligomers comprising an odd number of subunits and in facile subunit exchange), and (iii) the dimer interface is labile [128,129]. The evaluated thermodynamic association free energies of the edge and dimer interfaces (ΔGd, and ΔGe, respectively) of αB-crystallin and a comparison of the relative effects of alanine mutations at various position in the CTE on these free energies of association (ΔΔGd vs ΔΔGe) yields a negative correlation, indicating that destabilization of the C-terminal interaction stabilizes the intra-dimer interfaces [128]. It would be important to find out whether this observation with respect to αB-crystallin is also generally applicable to other sHsps, as this information would be useful in designing peptide-based inhibitors (with high binding affinity) for function of sHsps.

Thus, most of the work on the structural and chaperone functional relationship with respect to αB-crystallin (and to some extent with αA-crystallin) indicates a highly flexible assembly of the subunits with substrate-binding involving regions spanning all three, the NTD, ACD and the CTE. The prominent and most common sites appear to

correspond to the region comprising β-strands 3, 8 and 9, while other regions seem to make substrate-dependent contacts [61]. Identification of substrate-binding regions in other sHsps notably, Hsp27 and Hsp20 is required to understand the commonality or differences, if any, on the structural-functional aspects of sHsps in general. It emerges that the regions (strands 3, 8 and 9 and the β4-β8 groove) identified in αB-crystallin might be useful for targeting functionality of the sHsp.

1.9. Phosphorylation of sHsps

sHsps such as Hsp27, α-crystallins, Hsp20 and Hsp22 are known to get phosphorylated, especially under stress conditions. Phosphorylation of sHsps modulates a variety of their functions in cellular processes such as apoptosis, cell cycle and differentiation (which are discussed later wherever relevant). This section largely deals with the phosphorylation of sHsps and its role in oligomeric structure and chaperone property. A variety of stimuli leads to phosphorylation of serines(S), S15, S78, and S82 in human Hsp27, S15 and S86 in murine Hsp25, and S15 and S90 in Chinese hamster Hsp27 [130–132]. Mitogen-activated protein kinase (MAPK)-activated protein kinase 2 (MAPKAPK-2 or MK2 – substrate of the p38 MAPK) is a prominent kinase that phosphorylates Hsp27 [130, 133–136]. MK2 mediates the signaling events during inflammation, cell division and differentiation, apoptosis, and cell motility. Other kinases such as PKC (S15, S78), MK5 (S78, S82) and PKB and PKD (S82) are also known to phosphorylate Hsp27 (at the indicated sites) *in vivo* [reviewed in 131]. Phosphorylation of Hsp27 has been shown to be important for thermoprotection for cell survival, as the non-phosphorylable mimic of Chinese hamster Hsp27 does not offer such thermal protectivity [137].

Phosphorylation of Hsp27 *in vitro* and the mimicry of Hsp27 phosphorylation by mutations led to a decrease in the oligomeric size as well as the ability to prevent the thermal denaturation and refolding of citrate synthase *in vitro* [138]. On the other hand, a result of another study [137] shows very little difference in the chaperone-like activities of Chinese hamster wild type Hsp27, S15A/S90A-Hsp27 (the non-phosphorylatable form) and S15E/S90E-Hsp27- (the phosphorylation-mimic) towards the thermal aggregation of citrate synthase. It was observed that oligomer dissociation required only S90 phosphorylation of Chinese hamster Hsp27, while activation of thermoprotective activity required the phosphorylation of both S90 and S15 [137]. Using various deletion mutants and modeling studies, it was proposed that the region 5-23 (containing the conserved WD/EPF motif in mammalian Hsp27), which mediates important intramolecular interactions with hydrophobic surfaces of the α-crystallin domain of the protein, plays a crucial role in chaperone activity and thermoprotection [137]. These interactions and oligomerization are destabilized by S90 and S15 phosphorylation, making the motif free to interact with targets [137].

Dissociation of Hsp27 oligomer was a requisite for its recognition and binding to destabilized T4 lysozyme mutants [35]. Both phosphorylated Hsp27 and the phosphorylation mimic 3DHsp27 (S15D/S78D/S82D-Hsp27) show decreased oligomeric size and increased chaperone activity against DTT-induced aggregation of insulin or α-lactalbumin [140]. Interestingly, phosphorylation at different sites of Hsp27 has been reported to discriminate its target proteins [141,142, see reviews 139,143]: Using gel filtration chromatography to fractionate different Hsp27 species in HeLa cell lysate and immunoprecipitation strategies, it was shown that S15 phosphorylated Hsp27 (species less than 200 kDa) recognizes procaspase 3, while S82 phosphorylated Hsp27 (species of 400–700 kDa) recognizes histone deacetylase 6. Species of both S78 and S82 phosphorylated Hsp27 (200–700 kDa) are capable of recognizing STAT2 (signal transducer and activator of transcription 2) [142,143]. Further studies are required for understanding the role of phosphorylation of Hsp27 towards target recognition.

Both αA- and αB-crystallin are known to be phosphorylated. Various stimuli are known to induce phosphorylation, which may have implications in the regulation of their functions [144–146]. αA-crystallin has been shown to undergo cAMP-dependent kinase-mediated phosphorylation at S122. Three other phosphorylation sites between residues 122 and 173 have also been identified, but the kinase responsible is not known [147–149]. αB-crystallin has three phosphorylation sites – S19, S45 and S59 [144]. S19 phosphorylation occurs in an age-dependent manner. S45 phosphorylation is enhanced during mitosis of cells. S59 phosphorylation is known to occur under heat stress conditions [150]. While the kinase responsible for the phosphorylation of S19 is not known, p44/42MAP kinase has been shown to phosphorylate S45 and MAPKAP kinase-2 selectively phosphorylates S59 [150].

Phosphorylated αB-crystallin or its mimic 3DαB-crystallin (S19D/S45D/S59D-αB-crystallin) exhibit decreased oligomeric size compared to the wild type αB-crystallin [151]. It has been reported that phosphorylation of αB-crystallin in rat lens does not affect its chaperone activity [152]. The degree of protection offered by the 3DαB-crystallin to heat inactivation of luciferase in Chinese hamster ovary cells was found to be relatively less than that of αB-crystallin [151]. Phosphorylation-mimicking mutant exhibits less chaperone activity in preventing thermal aggregation of lactate dehydrogenase (LDH) [151] and aggregation of a model peptide ccβ-Trp peptide (a 17 amino acid model peptide that exists as a native-like coiled coil under ambient conditions but forms amyloid fibrils on increasing the temperature [153]) when compared to wild type αB-crystallin [34]. However, other studies have shown increased chaperone activity of phosphorylation-mimicking mutants or phosphorylated αB-crystallin compared to the wild type αB-crystallin towards many other target proteins. Phosphorylation-mimicking mutants of αB-crystallin exhibit increased chaperone activity in binding to the destabilized mutants of T4 lysozyme compared to the wild type protein [34], towards aggregation of insulin and α-lactalbumin (upon their disulfide reduction), heat stress-induced aggregation of catalase, alcohol dehydrogenase or β-crystallin and ordered aggregation of κ-casein [154]. A study from our laboratory [155] showed that 3DαB-crystallin exhibits higher chaperone activity towards heat-induced aggregation of citrate synthase, DTT-induced aggregation of insulin and the amyloid fibril formation of α-synuclein. A recent study showed that phosphorylation-mimicking mutant of αB-crystallin (3EαB-crystallin) exhibits decreased oligomeric size (to hexamer and 12-mer) and increased chaperone activity towards heat-induced aggregation of malate dehydrogenase and p53 compared to the wild type αB-crystallin [72].

Hsp20 is phosphorylated at S16 by cyclic nucleotide-dependent protein kinases. The phosphorylation-mimicking S16D mutation of Hsp20 did not affect the oligomeric status of Hsp20 but decreased the chaperone-like activity [26]. Though phosphorylation of Hsp22 *in vi*vo is not known, mammalian Hsp22 is shown to be phosphorylated by the cAMP-dependent protein kinase at S24 and S57 *in vitro* [156]. The S57D-Hsp22 mutant and the double mutant, S24D/57D-Hsp22 exhibited changes in the local environment of tryptophan residues and increased the susceptibility of Hsp22 to cleavage by chymotrypsin [156]. All the phosphorylation-mimicking mutants, particularly S24D/57D-Hsp22, exhibited decreased chaperone-like activity compared to that of Hsp22 [156]. Erk1 has also been shown to phosphorylate Hsp22 at S24, S27 and T87 *in vitro* [157]. The phosphorylation-mimicking mutations S24D, S27D and T87D, promoted concentration-dependent association of Hsp22. While S24D and S27D mutations decreased the chaperone activity of Hsp22, T87D mutation increased the chaperone activity of Hsp22 [157].

Thus, phosphorylation could modulate the oligomeric population and chaperone property of sHsps (Hsp27, αB-crystallin and Hsp22) *in vitro*. Dynamic exchange of subunits of the oligomers under equilibrium conditions gives rise to polydispersed distribution [see review 158]. Phosphorylation-mimicking mutants show increased rate of subunit exchange [72,155]. Increased dynamics of sHsps and/or decreased oligomeric size upon phosphorylation may increase the interactive species/surfaces with their clients and/or exposure of surfaces which are otherwise less/not accessible to the corresponding targets. Though phosphorylation of sHsps is observed *in vivo* under certain conditions, the

mechanistic insight of role of phosphorylation and its structural-functional relationship, especially in the *in vivo* context, is not completely understood. It is possible that nature uses phosphorylation as a means to either augment the function (increased interaction with the targets) or to switch between mutually exclusive functions (target discriminatory role/switching targets) of sHsps. Possibly the relative involvement of these functions may differ among sHsps. As phosphorylation is a potential handle to manipulate the function of sHsps, in depth understanding on this aspect is important for designing strategies for sHsps-mediated disease management.

1.10. sHsps and protein deposition diseases

Protein/polypeptide deposition diseases include mutated polyglutamine protein aggregation, Alexander's, Alzheimer's, Parkinson's, spongiform encaphalopathies, glial fibrillary acidic protein, type II diabetes, hereditary transthyretin amyloidosis etc., where disease-specific protein(s)/peptides misfold and aggregate in the form of fibrils termed amyloid fibrils [159]. sHsps have been demonstrated to bind to partially unfolded non-native states of proteins that are aggregation-prone and prevent their aggregation. Some misfolded proteins can overwhelm the chaperone and proteasomal degradation machinery, thus resulting in aggregation (often cytotoxic) of such proteins either as amyloid or amorphous deposits. Factors that can prevent aggregation of these proteins and facilitate their clearance could have therapeutic potential. Many sHsps have been found in the amyloid deposits [160–164].

Partially unfolded or partially folded (in the cases of natively unfolded proteins) species of proteins and peptide sequences with more hydrophobic residues, especially along with phenylalanine residues [165], have tendency to aggregate. Aggregation of proteins/peptides may either be ordered with intermolecular β-sheet formation called amyloid fibrils [159] or amorphous. Generally, amyloid fibril formation occurs with distinguishable nucleation (oligomers, short protofibrils etc.,) and elongation steps [166,167]. A critical balance between hydrophobic and electrostatic interactions is important in amyloid fibril formation [168]. In addition to their ability to prevent amorphous aggregation, many of sHsps also prevent amyloid fibril formation and cytotoxcity as discussed below.

Eye lens α-crystallin (composed of αA- and αB-crystallin) has been shown to prevent the amyloid fibrillar aggregation of proteins such as apolipoprotein C-II and serpin [169,170]. α-Crystallin interacts with partially structured amyloidogenic precursors of apolipoprotein C-II, suggesting that it inhibited the nucleation process of amyloid fibril formation [169]. Human Hsp27 and the murine Hsp25 have been shown inhibit the amyloid fibril formation of $A\beta_{1-42}$ [171].

A study from our laboratory showed that αB-crystallin prevented the fibril formation of $A\beta_{1-40}$ and $A\beta_{1-42}$ [172]. αB-crystallin did not form a stable complex with $A\beta_{1-40}$ peptide but it interacted with $A\beta_{1-40}$ fibril seeds, preventing the amyloid fibril growth of the Aβ peptide [172]. αB-crystallin prevented the spontaneous amyloid fibril formation of $A\beta_{1-42}$ peptide, indicating that αB-crystallin could also inhibit nucleation and/or elongation steps of fibril formation [172]. Based on the study we proposed [172] that α-crystallins can act on both nucleation and propagation (elongation) process of amyloid fibril formation, its relative involvement in these two phases of amyloid formation may depend on the nature of the amyloidogenic species.If the amyloidogenic species has significantly exposed hydrophobic surfaces, it may bind to this state and inhibit the nucleation process itself. On the other hand, α-crystallin could bind to the fibril nucleus, thus inhibiting the fibril elongation process [172]. Quartz crystal microbalance and analytical ultracentrifugation studies have also shown that αB-crystallin binds stably with the fibril nucleus [173]. αB-crystallin binds to wild-type $A\beta_{1-42}$ fibrils and also binds to fibrils of E22G Arctic mutation of $A\beta_{1-42}$ along the entire length and ends of the fibrils, inhibiting their elongation [173]. Experiments with single molecule detection approaches such as confocal two-color coincidence detection (cTCCD) and total internal reflection microscopy (TIRFM) have shown that αB-crystallin binds to Aβ oligomeric species, thereby preventing their growth into fibrils [174]. αB-crystallin, Hsp20 and Hsp27 inhibit aggregation of Aβ peptides and cerebrovascular Aβ toxicity [175]. Interestingly, αB-crystallin binds to cytotoxic oligomers of $A\beta_{1-42}$, islet amyloid polypeptide (IAPP), and the N-terminal domain of the prokaryotic hydrogenase maturation factor HypF (HypF-N) to form larger species, prevents the oligomer interaction with plasma membrane and decreases cytotoxicity [176].

The core ACD of αB-crystallin itself is able to prevent the amyloid fibril formation and the associated cytotoxicity of $A\beta_{1-42}$ with efficiency comparable to that of wild type αB-crystallin [103], indicating that the Aβ-interacting site most likely resides in the ACD of the protein. However, the core ACD of Hsp27 does not prevent the fibril formation of $A\beta_{1-42}$ [103], implying that most probably the NTD of Hsp27 contributes to its ability to prevent fibril formation of Aβ peptides.

α-Synuclein, which is a pre-synaptic, natively unfolded protein, assembles into a fibrillar form, which is a hallmark of several neurodegenerative diseases of α-synucleinopathies including Parkinson's disease [see reviews 177,178]. Studies from our laboratory [155] as well as from other laboratories [179–181] have shown that αB-crystallin prevents the amyloid fibril formation of α-synuclein. Other sHsps such as Hsp27, Hsp20, Hsp22 and HspB2B3-complex also prevent the fibril formation of α-synuclein, with Hsp22 being more potent [179]. Another study from our laboratory showed that HspB2 could also prevent the amyloid fibril formation of α-synuclein [30]. Transient interactions [179] as well as stable binding to fibril seed and prevention of elongation [179] have been implicated as mechanisms in preventing fibril formation of α-synuclein by sHsps.

The ability of sHsps to prevent amyloid aggregation and cytotoxicity appears to differ significantly depending on the amyloidogenic target. Hsp20, HspB7, Hsp22 and HspB9 inhibited polyglutamine protein aggregation effectively [182]; HspB7 and Hsp22 also inhibited cytotoxicity, not only in cells but also in a *Drosophila melanogaster* model [182]. On the other hand, Hsp27, αA- and αB-crystallin did not prevent the aggregation of mutated polyglutamine proteins. Interestingly, Hsp27 and αB-crystallin efficiently prevented the aggregation of mutated proteins that did not contain a polyglutamine stretch such as α-synuclein, GFAP and superoxide dismutase 1 [182]. Thus, these studies reiterate the notion that the ability of the sHsps to prevent aggregation of proteins also depends on the properties of the intermediate/aggregation-prone state(s) of proteins.

The physiological significance of binding of αB-crystallin (and other sHsps?) to oligomeric species and fibrils could be (i) preventing further elongation, (ii) preventing their cytotoxicity, (iii) stabilizing the fibrils against scission, therefore preventing the creation of multiple nucleation centers, and (iv) facilitating the clearance of inclusions by autophagy (described later). Interestingly, an isolated report shows that αB-crystallin binds to amyloid fibrils of apolipoprotein C-II, stabilizes the fibrils from dilution-induced fragmentation, prevents elongation of partially formed fibrils, and promotes the dissociation of mature fibrils into soluble monomers [183]. This interesting aspect of dissociation of fibrils by sHsp needs further investigation with different amyloidogenic proteins/peptides to unravel the underlying mechanism. One possible hypothesis is that binding of αB-crystallin to fibril ends affects the equilibrium between monomer at the fibril termini and the free monomer in favour of free monomer. It is also possible that binding of small molecules such as ATP and metal ions to sHsps complexed with fibrils or oligomeric species could modulate their ability, if any, to dissociate fibrils or oligomeric species. Focusing investigations on finding out whether other sHsps (for example Hsp27, HspB2, Hsp20) would also bind to oligomers and fibrils of amyloidogenic proteins/peptides would be useful in this regard.

1.11. sHsps and metal ions interactions

Human beings are commonly exposed to metals ions such as copper, cadmium, iron, aluminium and lead from various sources such as

industrial waste, air pollution, cigarette smoking, fossil fuel emissions and fertilizers. Toxic metal ions are known to be associated with oxidative stress, cell death and diseases. Accumulation of Cu^{2+} has been known to occur in genetic disorders such as Wilson's and Menke's disease [184,185]. Cu^{2+} has been implicated in several neurological disorders [186–188]. Treating lens epithelial cells with Cu^{2+} induces the expression of αA-crystallin, αB-crystallin and Hsp27, while Cd^{2+} induces the expression of αB-crystallin and Hsp27 [189]. These observations suggest that sHsps play a role in metal ions-associated processes. Cu^{2+} and Zn^{2+} significantly increase the chaperone-like activity of αB-crystallin towards the DTT-induced aggregation of insulin, while Ca^{2+} induces its aggregation [190]. However, the mechanism by which the metal ions induced increase in the chaperone-like activity of these sHsps is not known.

A study from our laboratory demonstrated, for the first time, that αA- and αB-crystallin bind Cu^{2+} with close to picomolar affinity, inhibit the Cu^{2+}-ascorbate-mediated generation of reactive oxygen species (ROS) and confer cytoprotectivity [191]. Binding of Cu^{2+} resulted in changes in their conformation and quaternary structures, and also led to increase in their stability to guanidine hydrochloride-induced denaturation [191]. Interestingly, Cu^{2+} promoted the aggregation of the presenile cataract-causing, folding-defective G98R mutant of αA-crystallin [192,193]. Both the N- and C- terminal domains contribute to the Cu^{2+}-binding, and redox-attenuating properties and Cu^{2+}-binding regions span the entire sequence of the protein [194]. Small-angle X-ray scattering and sedimentation velocity measurements showed increase in the oligomeric size, suggesting that a single oligomer of αB-crystallin can sequester a large number (~150) of Cu^{2+} ions (like a "Cu^{2+} sponge") [194]. At least one Cu^{2+}-binding site with picomolar affinity in the α-crystallin domain of αB-crystallin has been demonstrated [195]. NMR spectroscopic study showed that potential ligands coordinating Cu^{2+} were present in the loop regions connecting the β3 and β4 strands, and the β5 and β6 + β7 strands, and involved residues His83, His104, His111, and Asp109 [196]. These residues are well conserved among different metazoans as well as in human αA-crystallin, Hsp20 and Hsp27 [195]. Our recent study demonstrates that human Hsp27 also binds Cu^{2+} with close to picomolar affinity, inhibits the Cu^{2+}-ascorbate induced generation of ROS and confers cytoprotection [196]. Treating the human neuroblastoma cell line, IMR-32, with Cu^{2+} leads to up-regulation of endogenous Hsp27, and over expression of Hsp27 in these cells protects them from Cu^{2+}-induced cell death [196].

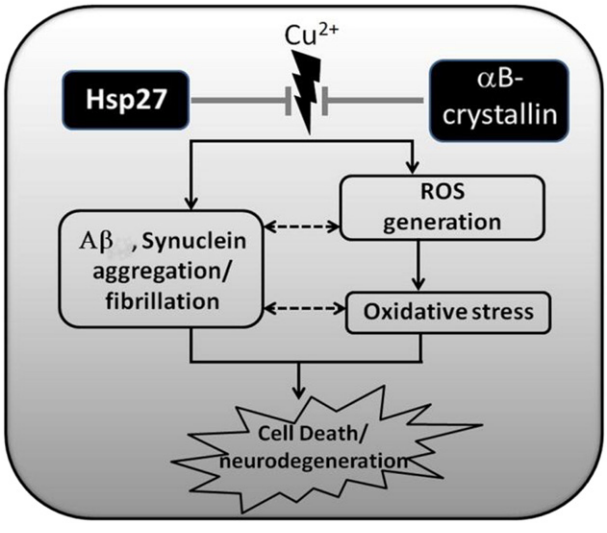

Fig. 2. Schematic representation of the protective role of sHsps in aberrant Cu^{2+} homeostasis [191,194,196]. Binding of Cu^{2+} by sHsps such as Hsp27 and αB-crystallin inhibits Cu^{2+}-mediated generation of ROS, oxidative stress, and amyloid aggregation/fibrillation of Aβ peptides and α-synuclein which subsequently leads to cytoprotective effects. These properties of sHsps have implications in neuroprotection under aberrant Cu^{2+} homeostasis conditions [191,194,196]. Scheme reproduced with modification from [196] with permission from Elsevier.

Considering increased expression of sHsps in neurodegenerative diseases [197,161–163], accumulation of sHsps in amyloid plaques [162–164], increased oxidative stress [186–188] and accumulation of the redox-active Cu^{2+} in the brain of patients suffering from Alzheimer disease [188], the Cu^{2+}-binding and redox attenuation of these sHsps has a protective role in Cu^{2+}-homeostasis and neurodegenerative diseases. Binding of Cu^{2+} to Aβ peptides, α-synuclein or prions is known to result in disease-causing deleterious aggregation, generation of ROS and cytotoxicity [186,187,198,199]. Fig. 2 schematically depicts the potential protective role of αB-crystallin and Hsp27 in Cu^{2+}-mediated oxidative stress, aggregation of Aβ peptide and α-synuclein and neurodegeneration. Aβ peptides bind Cu^{2+} and generate H_2O_2 [198], and Cu^{2+} induces aggregation of Aβ peptides [199]. α-Crystallins [191] and Hsp27 [196] prevent the Cu^{2+}-induced aggregation of $Aβ_{1-40}$. Hsp27 [196] and αB-crystallin (our unpublished results) prevent the Cu^{2+}-induced amyloid fibril formation of α-synuclein by dislodging the Cu^{2+}-bound to α-synuclein, rendering it less amyloidogenic [196]. Cu^{2+} and ascorbate-mediated generation of ROS is a potential means of oxidative stress, given that the levels of ascorbate can reach as high as 0.4 mM in brain extracellular fluid and to ~10 mM in neurons [200], while Cu^{2+} can reach ~0.4 mM in the brains of patients with Alzheimer's disease [188]. α-Crystallins and Hsp27 may play protective role in the process of Cu^{2+}-induced aggregation of Aβ peptides and α-synuclein as well as Cu^{2+}-induced oxidative stress [191,196]. Since sHsps such as αB-crystallin, Hsp27, HspB2, HspB3 and Hsp22 are also found in the plaques of Aβ peptides in the brains of patients of Alzheimer's disease and in the Lewy bodies in patients with Parkinson's disease [160,161,201], investigating the binding of metal ions by these sHsps, their role in the Cu^{2+} homeostasis and in these disorders, if any, would give insights into this novel cytoprotective function of sHsps.

α-Crystallins also interact with metal ions such as Ca^{2+} and Zn^{2+}. α-Crystallin, the major eye lens protein (composed of αA- and αB-crystallin), is involved in maintaining lens transparency. High levels of Ca^{2+} (as high as 64 mM) are associated with cataract formation [202]. Ca^{2+} decreases the anti-aggregation activity and thermal stability of α-crystallin [203,204]. The impaired chaperone activity would reduce its ability to prevent aggregation of lens protein, thus playing a role in progressive loss of transparency and cataractogenesis [203,204]. Zn^{2+} interacts with α-crystallin with dissociation constant in the sub-millimolar range, increases its surface hydrophobicity, increases its stability to urea denaturation as well as resistance to trypsin digestion and enhances its chaperone activity by 30% [205]. Further studies are required to understand the effect of metal ions on the structure and functions of sHsps and their role in pathology involving aberrant metal ions homeostasis.

1.12. Hetero-oligomer formation between the subunits of sHsps

sHsps exchange their subunits to form hetero-oligomeric complexes [41,42,206–208]. αB-crystallin co-purifies with Hsp27 from skeletal muscle [209]. They associate with each other *in vivo* and form hetero-oligomers *in vitro* [208–210]. αA- and αB-crystallin form hetero-oligomers *in vitro* [41] and their hetero-oligomers (at 3:1 ratio respectively) are isolated as α-crystallin from the eye lens [see reviews 211, 212]. Two types of hetero-oligomeric complexes – one consisting of HspB2 and HspB3 and the other of Hsp27, αB-crystallin and Hsp20 have been found in muscle tissue [213]. Using various techniques including gel-filtration chromatography, immunoprecipitation, yeast two hybrid assay and FRET microscopy, Hsp22 was found to interact with itself, HspB7, HspB2 and Hsp27 [214]. Using yeast two hybrid assay and FRET microscopy, Hsp22 has been found to interact with Hsp20, and αB-crystallin [215]. However, a cysteine mutant of Hsp22 corresponding to the C137 position of Hsp27 did not form cross-linked hetero-dimers with other sHsps such as Hsp27, αB-crystallin

and Hsp20 efficiently [216]. On the other hand, similar cysteine mutants of αB-crystallin and Hsp20 formed cross-linked hetero-dimers with each other and with Hsp27 efficiently [216]. It appears that Hsp22 interactions with other sHsps warrant further investigation for clear understanding. While the formation and existence of hetero-oligomeric species of two or more different sHsps occurs *in vitro* and *in vivo*, the exact structural and functional consequences and their biological significance have not been completely understood. However, formation of hetero-oligomers between sHsps modulates their chaperone activity.

A study from our laboratory [23] showed that the chaperone activity of isolated bovine αB-crystallin was higher than that of αA-crystallin and lens α-crystallin at lower temperatures, but the activity of αA-crystallin and lens α-crystallin increased at higher temperatures. αA-crystallin was found to be more stable to temperature-induced changes than αB-crystallin, suggesting that the hetero-oligomers of αA- and αB-crystallin present in the mammalian eye lens (αA-: αB-crystallin: 3:1 ratio) may be of importance in providing appropriate stability and optimal chaperone-like activity [23]. Investigation of the structural and chaperone activity changes during the course of hetero-oligomer formation by the exchange of subunits between homo-oligomers of αA- and αB-crystallin at various ratios shows that hetero-oligomers display differential chaperone-like activity which correlate with subunit exchange and the resulting large tertiary and quaternary structural changes [41]. A study from another laboratory has shown that αA-crystallin in its hetero oligomer formed *in vitro* with αB-crystallin imparts stability [217].

The hetero-oligomers of Hsp27 and αB-crystallin were intermediate in terms of size and stability to heat stress relative to the two homo-oligomers [208]. Hsp27 is an inferior chaperone, whereas αB-crystallin is the determining factor of the hetero-oligomers in the suppression of both amorphous and ordered aggregation of target proteins [208]. The rate of subunit exchange between Hsp27 and αB-crystallin, as monitored by mass spectrometry, was 23% faster than that between Hsp27 and αA-crystallin and twice as fast compared to that between αA- and αB-crystallin [208]. Co-expression of αB-crystallin and Hsp27 in HeLa cells led to increase in phosphorylation of Hsp27 and increase in the activity of glucose-6-phosphate dehydrogenase (whose activity is increased by smaller sized phosphorylated Hsp27), indicating that potential hetero-oligomer formation between Hsp27 and αB-crystallin can indirectly modulate Hsp27 phosphorylation and function [218]. HspB2 and HspB3 form a series of well defined hetero-oligomers, consisting of 4, 8, 12, 16, 20 and 24 subunits *in vitro*, in which HspB2 and HspB3 are present in a 3:1 subunit ratio [207]. The HspB2-HspB3 complex exhibited poor chaperone-like activity [207]. Studies from our laboratory [30,31] showed that the homo-oligomers of HspB2 and HspB3 exhibited target protein-dependent chaperone activity. Thus, it appears that the hetero-oligomers of HspB2-HspB3 acquire different activity from that of their respective homo-oligomers.

Besides modulation of chaperone activity, hetero-oligomer formation can also regulate other, yet unclear, cellular processes. Hsp20 and Hsp27 form two different hetero-oligomeric species of molecular masses 100-150 kDa (lower concentration) and ~250–300 kDa (higher concentration) with a stoichiometry of 1:1 [219]. Upon hetero-oligomer formation with Hsp27, the rate of phosphorylation of Hsp20 by cAMP-dependent protein kinase was inhibited [219]. Hsp20 inhibited the MAPKAP-kinase 2 phosphorylation of Hsp27 upon hetero-oligomer formation [219]. Thus, hetero-oligomer formation between Hsp27 and Hsp20 can regulate phosphorylation events and hence the consequent processes.

Hsp20 and αB-crystallin were found to be co-immunoprecipitated from heart homogenates [220]. Immunofluorescence microscopy showed that staining of Hsp20, αB-crystallin, and actin was predominantly in transverse bands – αB-crystallin and Hsp20 were associated at the level of the actin sarcomere [220]. Phosphorylated Hsp20 as well as phosphopeptide analogues of Hsp20 increased myocyte shortening rate. However, it is not clear whether myocyte shortening is brought about exclusively by the hetero-oligomer or by the homo-oligomers of αB-crystallin and Hsp20 and how the disruption of the hetero-oligomer, if formed, affects myocyte shortening. A 700 kDa complex of Hsp27 and αB-crystallin was found to be present in non-oncogenic cells but not in oncogenic cells [221]. The complex falls apart upon heat stress, but forms again upon recovery. It would be interesting to find out the significance of the absence of this complex in oncogenic cells, and whether it serves a discriminatory purpose for the immune system to eliminate tumor cells.

Point mutations in sHsps seem to alter inter-sHsp interactions. The interactions of the congenital cataract causing mutant, R116C αA-crystallin with αB-crystallin and Hsp27 increased almost 5-fold over those with the wild type protein [222]. Interaction of R120G αB-crystallin (which causes desmin-relation myopathy and congenital cataract) with αA- and αB-crystallin is decreased compared to that with the wild-type protein [222]. The αB-crystallin mutants, R120G, Q151X and 464delCT exhibited abnormal interactions with Hsp20, whereas the R120G and Q151X mutants exhibited altered interactions with Hsp22 [223]. Using cyan (CFP) and citrine (CIT) fluorescent protein tagged sHsp constructs, it was shown that K141E or K141N mutants of Hsp22 (involved in inherited peripheral motor neuron disorders distal hereditary motor neuropathy type II and axonal Charcot-Marie-Tooth disease type 2 L) exhibit aberrantly increased interactions with themselves, wild-type Hsp22, αB-crystallin and Hsp27, but not with Hsp20 [224]. The S135F mutation in Hsp27 (also associated with these disorders) exhibits increased interaction with wild-type Hsp22 [224].

Thus, mutations in sHsps alter the apparent inter-sHsp interactions. It is possible that alteration in the inter-sHsp interactions leads to absence of the original interactions of the sHsp. It also acquires deleterious gain of interaction of the mutant protein with the new partner. As mutations alter native conformation, often increasing the aggregation propensities, some of the increased interactions of the mutants with wild type sHsps are likely to be of the less-specific type chaperone-target protein interactions through exposed hydrophobic surfaces rather than specific replacement of subunits in the sHsp assemblies through subunit exchange. Developing methods to distinguish these two types of interactions among the mutant and wild type sHsps is important to understand how alterations of inter-sHsp-interactions are involved in pathological conditions.

1.13. Interactions of sHsps with cytoskeletal and nucleoskeletal elements

sHsps can interact and thereby affect the spatio-temporal organization of the cytoskeletal components. αB-crystallin, Hsp27 and Hsp20 are present in high concentrations in vascular smooth muscle cells [see review 225]. αB-crystallin and Hsp27 interact with intermediate filaments, stabilize them and prevent their reorganization [226,227]. Immuno-precipitation experiments with lysates of L6 myoblast cells demonstrated the association of αB-crystallin with tubulin; αB-crystallin prevented the aggregation of tubulin *in vitro* [228]. αB-crystallin is known to associate with desmin and chaperone it [226]. αB-crystallin can bind to and stabilize microtubules [229]. Hsp27 [230, 231] and αB-crystallin [232] bind and stabilize F-actin and prevent its depolymerization. The phosphorylation mimic of Hsp27 has been shown to prevent aggregation of actin upon thermal stress by binding to form soluble complexes [233].

Studies from our laboratory [232] have shown that under conditions of heat stress, αB-crystallin associates with actin fibers in H9C2 rat cardiomyoblast cells in a S59 phosphorylation-dependent manner providing resistance to cytochalasin B-induced actin disorganization. Murine Hsp25 has been reported to inhibit actin polymerization [234]. Hsp20 is expressed both constitutively and inducibly in airway smooth muscle and its cAMP-dependent protein kinase phosphorylated form promotes airway smooth muscle relaxation [235]. Endogenous αB-crystallin and Hsp25 of H9C2 rat cardiac myoblasts as well as ectopically

expressed αA-crystallin, Hsp20, HspB2 and HspB3 have been shown to associate with actin cytoskeleton upon proteasomal inhibition [236].

A study from our laboratory [237] has shown that upon subjecting C2C12 myoblasts to heat stress, αB-crystallin and Hsp25 translocated to the nucleus, where they co-localized with the intracellular lamin A/C and the splicing factor, SC-35 in the nuclear speckles. However, their role within the nucleus is largely unaddressed; presumably they may affect the stability of the nuclear components. They relocated to the cytoplasm upon recovery. Studies from other laboratories have shown that pseudophosphorylated αB-crystallin also translocated to the nucleus and colocalized with the splicing factor SC-35 in the nuclear speckles [238,239]. FBX4, an adapter protein of the ubiquitin-protein isopeptide ligase SKP1/CUL1/F-box, was also recruited to the nuclear speckles, suggesting that αB-crystallin had a phosphorylation-dependent role in the ubiquitination of a protein component of the nuclear speckles [238,239]. It is important to investigate further the significance of interaction of sHsps with nucleoskeletal elements.

As mentioned earlier, R120G mutation in αB-crystallin causes desmin-related myopathy and congenital cataract. Lens fiber cells do not have desmin, but have vimentin, CP 49 and filensin. R120G αB-crystallin promoted the aggregation of vimentin in the lens, indicating that increased interaction of the mutant protein with vimentin and the resulting aggregation was the molecular basis for congenital cataract [240]. R116C αA-crystallin interacted significantly less with actin than wild type αA-crystallin [241]. Since both αA-crystallin and actin are necessary for the proper development of lens, decreased interaction of mutant αA-crystallin may perturb the normal differentiation process of lens cells that is necessary for the lens transparency [241]. αB-crystallin has been shown to bind to myofibrils in cardiac muscle under conditions of ischemia. It binds to titin in the I-band of cardiac fibers [242]. αB-crystallin binds to a discrete region of the I-band (in the N2B region of the protein, titin) that moves away from the Z-disc when sarcomeres are extended [243]. αB-crystallin bound to the immunoglobulin region of titin between the N2B domain and the Z-disc and stabilized it. The association of αB-crystallin with the I-band titin prevented stress-induced unfolding [243]. Thus, sHsps are important in maintaining cytoskeletal integrity.

1.14. Anti-apoptotic function of sHsps

αB-crystallin prevents cell death induced upon oxidative stress and upon treating with drugs such as staurosporin and doxorubicin [11,244–246]. Both human αB-crystallin and Hsp27 have been shown to prevent TNF-α-induced apoptosis – over-expression of these proteins leads to increased levels of glutathione and decrease in TNF-α-induced increase in ROS [245]. L929 cells expressing either Hsp27 or αB-crystallin are resistant to apoptosis induced by staurosporine, an inhibitor of kinase C [246]. Such activity of these sHsps, which results in increased levels of glutathione, thus, can indirectly contribute in preventing cell death pathways triggered by high levels of ROS generation. Similarly, the ability of sHsps to interact with cytoskeletal elements and protect them under conditions of stress can also prevent the cascade of events leading to cell-death. There seems to be an apoptotic signaling pathway linking cytoskeletal damages to mitochondria [231]. The F-actin depolymerizing agent, cytochalasin D, induces the release of cytochrome c from mitochondria and subsequent activation of caspase. The phenomenon was delayed in cells pretreated with phalloidin, an F-actin stabilizer. Over-expression of Hsp27 leads to the intracellular relocalization of Bid (and delayed release of cytochrome c from mitochondria) upon treatment with cytochalasin D, indicating that Hsp27 interferes with apoptotic signals upstream of the mitochondrial apoptotic pathway [231].

Some sHsps have been shown to be involved in various stages in inhibiting mitochondria-mediated apoptosis (Schematically depicted in Fig. 3). Subjecting lens epithelical (HLE-B3) and a retinal pigment epithelial cell line (ARPE-19 cells) to oxidative stress leads to translocalization of αB-crystallin to the mitochondria and protection of the mitochondrial membrane potential [247]. αB-crystallin interacts with the pro-apoptotic molecules, Bax and Bcl-Xs and prevents apoptosis by inhibiting their translocation into mitochondria [248]. Hsp20 protects heart from ischemia and reperfusion injury-induced necrosis and apoptosis, improves recovery of cardiac function and reduces infarction [249]. Hsp20 interacts with the pro-apoptotic protein Bax, preventing its translocation from the cytosol to mitochondria, leading to decreased caspase-3 activation [249]. Phosphorylation at S16 residue of Hsp20 is shown to be important for its anti-apoptotic and cardioprotective function [250]. Enhanced expression of Hsp27 indirectly inhibited Bax activation, oligomerization, and translocation to mitochondria, reducing the release of both cytochrome c and apoptosis-inducing factor [251]. Hsp27 expression prevented the inactivation of Akt, a pro-survival kinase, and increased the interaction between Akt and Bax, an Akt substrate during metabolic stress. Thus, Hsp27 antagonizes Bax-mediated mitochondrial injury and apoptosis by promoting Akt activation via a PI3-kinase-dependent mechanism [251].

αB-crystallin interacts with cytochrome c and protects it against its oxidation at the M80 residue [247]. Hsp27 inhibits cytochrome c-mediated activation of caspases in the cytosol by interacting with cytochrome c and prevents cytochrome-c-mediated interaction of Apaf-1 with procaspase-9 [252,253]. Hsp27 also prevents cytochrome c and dATP-triggered activity of caspase-9, downstream of cytochrome c release [254].

Hsp27 also prevents apoptosis by inhibiting the release of the second mitochondria-derived activator of caspases, Smac, which promotes apoptosis via activation of caspases [255]. Over-expression of αB-crystallin in RPE cells protects from ER stress-induced apoptosis by attenuating increases in Bax, CHOP, mitochondrial permeability transition, and cleaved caspase-3 [256].

Silencing αB-crystallin sensitized ARPE-19 cells to methyl glyoxal (MGO)-induced apoptosis [257]. αB-crystallin interacts with the caspase subtypes, caspase-2L, -2S, -3, -4, -7, -8, -9 and -12 in control ARPE-19 cells, and MGO treatment leads to dissociation of caspase subtypes from αB-crystallin [257]. Phosphorylation of αB-crystallin on serine residues 19, 45 and 59 plays an important role in preventing apoptosis in ARPE-19 cells [257]. However, it has also been reported that S59-phosphorylated-αB-crystallin preferentially interacts with and sequesters the anti-apoptotic molecule, Bcl-2 and hence S59-phosphorylation down-regulates the anti-apoptotic function of αB-crystallin [258]. αB-crystallin suppresses apoptosis also by binding to the pro-caspase-3 and the p24 processing intermediate of caspase-3 and preventing its maturation to the proteolytically active enzyme [259,260]. Hsp27 is also shown to bind to pro-caspase-3 [253,261,262].

Hsp27 also plays a role in the regulation of cellular senescence by modulating the p53 pathway. The tumour suppressor, p53, is involved in essential functions such as DNA repair, transcription, genomic stability, senescence, cell cycle control and apoptosis [263]. Hsp27 is one of the target genes of p53 and p53-dependent induction of Hsp27 expression has been observed [264]. Hsp27 inhibits p53-mediated induction of p21/Waf1, an inhibitor of cyclin-dependent kinases and the major regulator of the senescence program. Hsp27 inhibited accumulation of p21 and suppressed senescence in response to the p53 activator nutlin-3, indicating that Hsp27 has a general effect on the p53 pathway [265]. Over-expression of a phosphorylation-mimic of Hsp27 was shown to result in the activation of p53/p21 in an ATM (Ataxia Telangiectasia Mutated)-dependent manner [266]. Hsp27 phosphorylation was found to increase the import of p53 into the nucleus and the expression of p53 target genes p21 and MDM2. Inhibition of Hsp27 phosphorylation was found to reduce p53 induction and p21 accumulation, leading to apoptosis [266]. Thus, Hsp27, and its phosphorylation status, plays an important role in regulating the p53 pathway and cell survival. αB-crystallin has also been shown to interact with p53 in the cytoplasm during hydrogen peroxide-induced apoptosis [267].

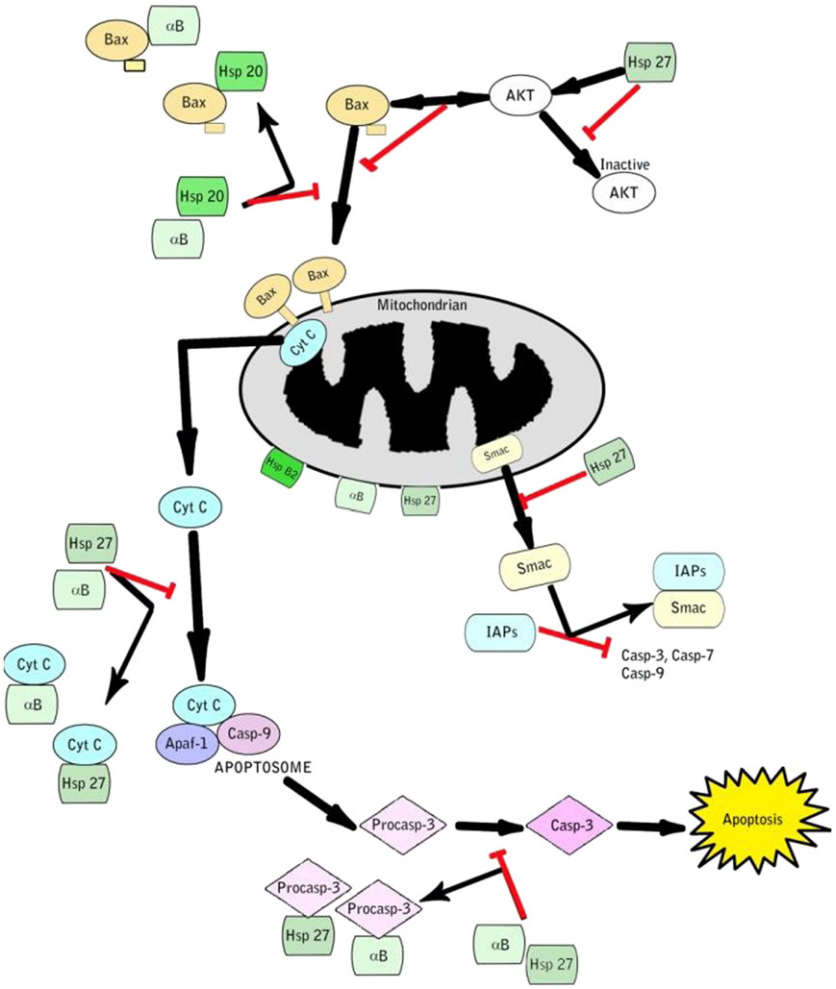

Fig. 3. Schematic diagram representing anti-apoptotic functions of sHsps at various stages of mitochondrial apoptotic pathways. αB-crystallin and Hsp20 interact with the pro-apoptotic molecule, Bax and inhibit its translocation into mitochondria and the subsequent release of cytochrome c [248,249]. Hsp27 prevents the inactivation of Akt, a pro-survival kinase, increases the interaction of Akt with its substrate, Bax, inhibiting Bax activation, oligomerization and translocation to mitochondria, thus inhibiting the release of cytochrome c (as well as apoptosis-inducing factor, not shown) [251]. αB-crystallin and Hsp27 interact with cytochrome-c and prevent cytochrome-c-mediated interaction of Apaf-1 with procaspase-9 to form apoptosome [247,252,253]. Hsp27 inhibits the release of the second mitochondria-derived activator of caspases, Smac, which binds to the inhibitor of apoptosis proteins (IAPs) and promotes apoptosis via activation of caspases [255]. αB-crystallin and Hsp27 interact with procaspase-3 and prevent its maturation to active caspase-3 [253,257,259–262]. αB-crystallin, HspB2 and Hsp27 are also found to localize to mitochondria [247,274,275], probably protecting their integrity and viability. The exact significance of such mitochondrial localization of sHsps is still to be explored. The scheme represents interactions of sHsps with factors on the apoptotic pathway shown mostly by pull-down or immunoprecipitation methods and therefore does not indicate stoichiometry or distinguish between direct and indirect interactions.

Some sHsps also act on the extrinsic pathway, the receptor(s)-mediated initiation of apoptotic events (Schematicaly depicted in Fig. 4). Hsp27 inhibits apoptosis induced by the activation of the death receptor Fas upon exposure to anticancer drugs. Expression of Hsp27 in L929 cells that express the cell surface Fas/APO-1 receptor prevents the apoptosis induced by agonistic anti-APO-1 antibody [246]. Two apoptotic pathways can be activated downstream of Fas- (i) A caspase-dependent pathway mediated by the Fas-adaptor FADD, and (ii) the caspase-independent pathway - the interaction of Fas with cytoplasmic Daxx and apoptosis signal-regulating kinase (ASK1) triggering apoptosis. Fas activation also induces translocation of Daxx from the nucleus (nuclear Daxx) to the cytoplasm. Hsp27 has been shown to act at the latter pathway [12]. Phosphorylated dimers of Hsp27 interact with Daxx, preventing its interaction with ASK1 and Fas, thus inhibiting Daxx-mediated apoptosis [268]. Hsp27 also prevents the translocation of nuclear Daxx to the cytoplasm. ASK1-mediated pathway plays important roles in oxidative stress- and endoplasmic reticulum (ER) stress-induced apoptosis [269]. Activation of the kinase ASK1 leads to the activation of JNK via MKK4 and MKK7, resulting in apoptosis in response to oxidative stress, endoplasmic reticular stress, DNA damage, or inflammation [270]. The NTD of Hsp27 is known to physically interact with the kinase domain of ASK-1, effectively inhibiting its activity [271]. Thus, Hsp27 inhibits ASK1-mediated cell death pathway and confers neuroprotection [271,272]. Phosphorylation of Hsp27 (mediated by protein kinase D) critically at S15 and S82 is important for neuroprotection [272].

HspB2 has been reported to be a negative regulator of the caspase-dependent extrinsic apoptotic pathway [273]. Breast cancer cells stably expressing HspB2 are resistant to apoptosis induced by TRAIL and TNF-α. HspB2 inhibits the most proximal step in the extrinsic apoptotic pathway, proteolytic activation of initiator caspases-8 and -10 [273]. Activated caspase-8 and -10 can cause apoptosis either by activating caspase-3, or by truncating Bid (to t-Bid), subsequently triggering its translocation to mitochondria and initiating the mitochondrial apoptotic pathway [276]. The exact mechanism by which HspB2 inhibits the activation of caspases-8 and -10 is not known. It is possible that HspB2 may disrupt recruitment of both the adaptor protein FADD and procaspases-8 and -10 to ligand-bound death receptor complexes [273]. HspB2 is also shown to be localized to mitochondria [274]. At higher levels of expression, HspB2 inhibits tBid-induced apoptosis, suggesting that HspB2 weakly inhibits mitochondrial or post mitochondrial apoptotic events [273]. It is likely that interaction of HspB2 with

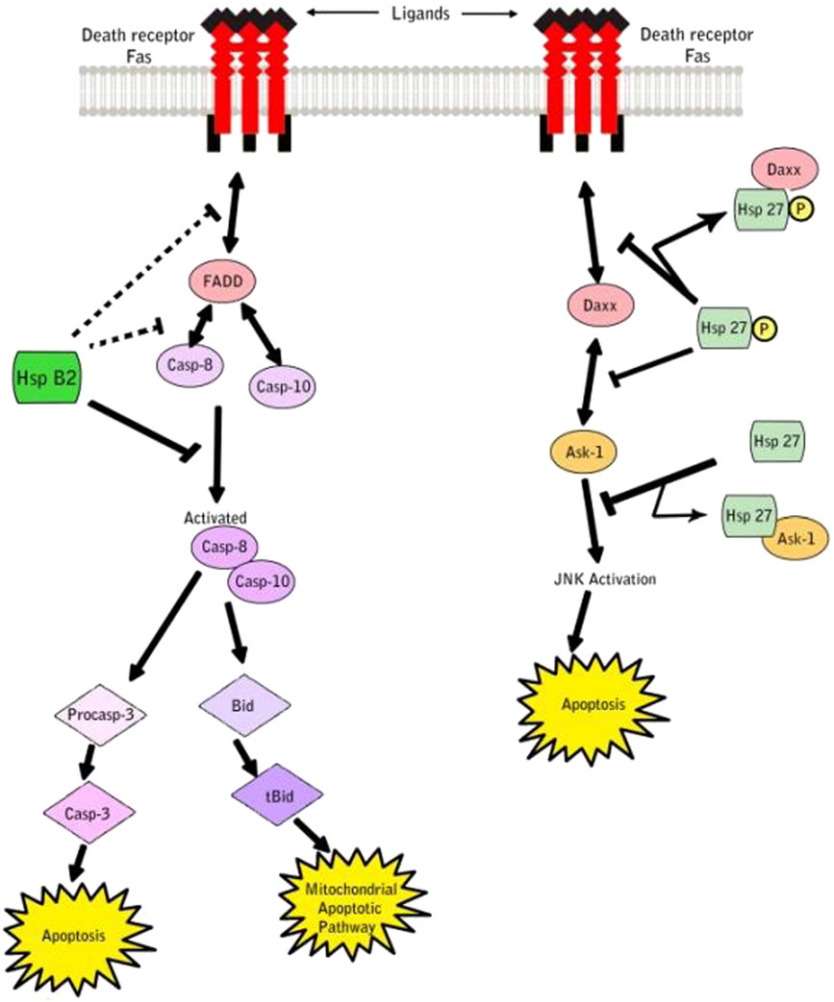

Fig. 4. Schematic diagram representing the role of sHsps in the extrinsic apoptotic pathway through the membrane located Fas stimulation upon its external ligand binding – (i) HspB2 interferes in the caspase-dependent extrinsic apoptotic pathway mediated by the Fas-adaptor, Fas associated death domain, FADD [273]. Engagement of FADD and pro-caspase-8 and -10 leading to activation of caspase-8 and -10, which can either activate caspase-3 resulting in apoptosis, or activate Bid by truncation subsequently triggering its translocation to mitochondria and initiation of mitochondrial apoptotic pathway [276]. HspB2 prevents activation of caspase8 and -10 [273], but the exact mechanism as to whether it interferes (shown as dashed lines) in interaction of the procaspase-8 and -10 with FADD or in the interaction of FADD with the Fas is not known. (ii) Interaction of Fas (stimulated by its ligands) with cytoplasmic Daxx and apoptosis signal-regulating kinase (ASK1) triggers apoptosis. Phosphorylated dimers of Hsp27 (at S15 and S82) interact with Daxx, preventing its interaction with ASK1 and Fas, thus inhibiting Daxx-mediated apoptosis [277]. Activation of the kinase ASK1 leads to the activation of JNK via MKK4 and MKK7, resulting in apoptosis in response to oxidative stress, endoplasmic reticular stress, DNA damage, or inflammation [270]. The N-terminal domain of Hsp27 interacts with the kinase domain of ASK1, inhibiting its activity [271]. The scheme represents interactions of sHsps with factors on the apoptotic pathway shown mostly by pull-down or immunoprecipitation methods and therefore does not indicate stoichiometry or distinguish between direct and indirect interactions.

mitochondria has a role to play in energy metabolism as well. Recovery of ATP concentration during reperfusion after ischemia was found to be impaired in HspB2-knockout mice [278]. Upon inotropic stimulation, an HspB2-knockout mouse was found to have blunted systolic and diastolic function, revealing massive energy wasting on acute stress [278].

A study from our laboratory [279] has shown that αB-crystallin exhibits anti-apoptotic function against TNF-α-induced apoptosis through activation of the ubiquitous transcription factor, NF-κB, leading to increased expression of anti-apoptotic protein(s), for example, Bcl-2 in mouse myoblast cells (C2C12). This constitutes yet another mechanism by which αB-crystallin prevents cell death (Schematically depicted in Fig. 5). This process appears to be important in muscle homeostasis. αB-crystallin knockout mice die prematurely with extensive muscle wastage [277]. The level of αB-crystallin is increased about 10-fold during muscle differentiation and it plays an anti-apoptotic role during the differentiation process [10,260]. αB-crystallin inhibits maturation/activation of executor caspases such as caspase-3 and -8 in response to TNF-α treatment, preventing cellular responses to apoptosis [244]. TNF-α is an inflammatory cytokine produced by immune cells and muscle cells during development and exercise or injury [280]. TNF-α acts as a mitogen in skeletal muscle [280,281]. It modulates the activity of NF-κB which regulates the expression of many genes [282]. NF-κB is activated through a cascade of events and its activation mediates pathways related either to cytoprotection or cell death [283], probably depending on conditions that are not very well understood. Expression of αB-crystallin as well as TNF-α increases in response to stress and differentiation [10,280]. Owing to its inflammatory effects, TNF-α may trigger cytotoxic effects upon prolonged exposure but is required during the differentiation process. The elevated expression of αB-crystallin could be the balancing protective process. In order to understand the relation between the elevated expression of αB-crystallin and TNF-α, a study from our laboratory [279] investigated the effect of over-expression of αB-crystallin in C2C12 mouse myoblasts on TNF-α-induced cell death and modulation of NF-κB activation. The study shows that αB-crystallin promotes NF-κB activation in a phosphorylation-dependent manner and protects myoblasts against TNF-α induced cytotoxicity [279]. αB-crystallin interacts with IKKβ upon treating the cells with TNF-α. Such interaction enhances the kinase activity of IKKβ, which leads to phosphorylation and subsequent degradation of IκB-α, a negative regulator

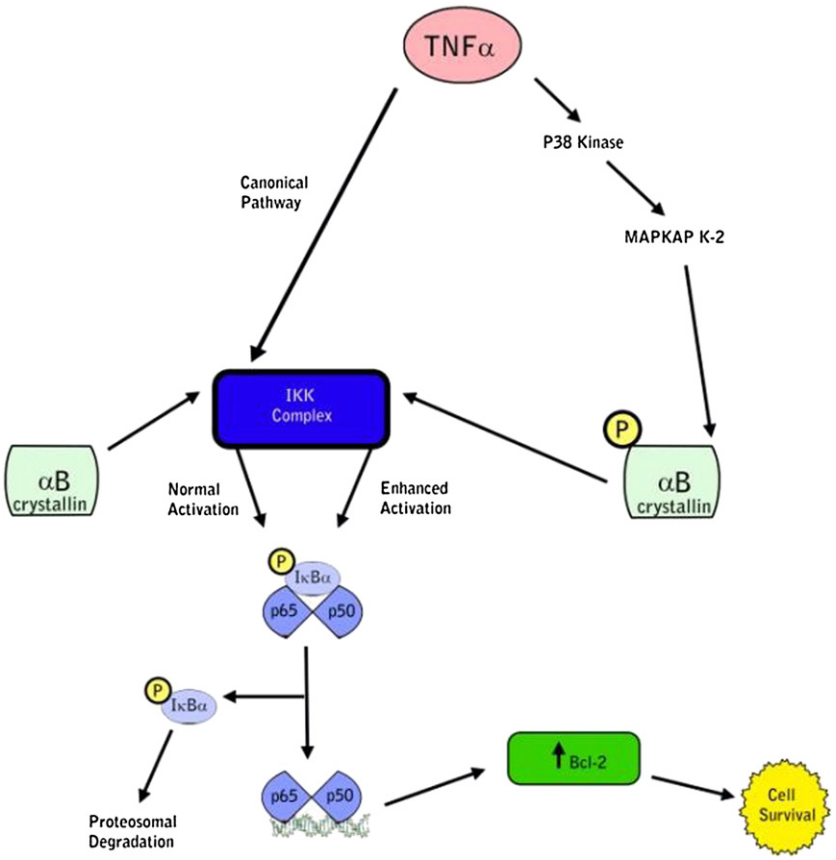

Fig. 5. Schematic representation of the cytoprotective role of αB-crystallin in TNF-α-induced apoptosis through activation of NF-κB pathway [279]. TNF-α increases phosphorylation of αB-crystallin at S59 through p38-MAP kinase pathway. αB-crystallin or its phosphorylated form associates with and enhances the kinase activity of IKK complex, thereby facilitating phosphorylation and subsequent degradation of IκB, leading to nuclear translocation and transcriptional activation by NF-κB-p65-protein. Under the condition, elevated expression of Bcl 2, an anti-apoptotic protein (a known target of NF-κB pathway) occurs which confers protection to cells against TNF-α-induced cytotoxicity. The scheme represents interactions of sHsps with factors on the apoptotic pathway shown mostly by pull-down or immunoprecipitation methods and therefore does not indicate stoichiometry or distinguish between direct and indirect interactions.

of NF-κB, facilitating the nuclear translocation of the transcription factor. One of the targets of such αB-crystallin-induced activation of NF-κB is the transcriptional up-regulation of the anti-apoptotic protein, Bcl-2; thus, αB-crystallin protects the cells from TNF-α-induced cytotoxicity [279]. Phosphorylation of αB-crystallin at S-59 residue is essential in the activation NF-κB [279]. Incidentally, TNF-α also activates p38-MAP kinase [284], which is responsible for the phosphorylation of αB-crystallin at S-59 residue [144]. A study from our laboratory has shown that S-59 phosphorylation is not required for association of αB-crystallin with the IKK complex [279]. However, it is essential for enhancing the kinase activity of IKKβ [279]. Thus, αB-crystallin exhibits anti-apoptotic function through its interactions with various client proteins involved at various stages of the apoptotic pathways.

As a deviation from the general anti-apoptotic and pro-survival properties of the sHsps discussed above, Hsp22 appears to have dual roles. The levels of Hsp22 and its mRNA were found to be decreased in melanoma, sarcoma and prostate cancer compared to normal cells, whereas it is abundantly expressed in proliferative keratinocytes [285]. Induced expression of Hsp22 by the demethylating agent, 5-aza-2'-deoxycytidine, in cancer cells where it is down-regulated, triggers apoptosis by caspase- and p38MAPK-dependent pathways [285]. However, heat-induced over-expression of Hsp22 did not result in increased apoptosis in HEK293 cells [285]. It is possible that the anti-apoptotic stimuli exerted by the over-expression of other heat shock proteins upon heat shock may overwhelm the pro-apoptotic stimuli of Hsp22; it is also possible that the pro-apoptotic property of the protein is specific to the demethylating agent and/or cell type-specific.

The viral homologue of Hsp22, ICP10 PK confers neuroprotection and prevents apoptosis in cultured hippocampal neurons by activating the extracellular signal-regulated kinase (ERK) survival pathway, resulting in the up-regulation of the anti-apoptotic protein, Bag-1 as well as increased activation/stability of the transcription factor CREB and stabilization of the anti-apoptotic protein Bcl-2 [286]. Hsp22 offers cardioprotection from ischemic reperfusion injury and exhibits anti-apoptotic property by activation of the Akt (also known as Protein Kinase B) pathway (see review [287]). Hsp27 is also shown to play a role in Akt/ERK pro-survival pathway. Phosphorylation of Hsp27 at S78/82 residues was found to be essential for TRAIL-triggered Src-Akt/ERK signaling [288]. Hsp27 regulates Akt activation by binding and mediating interaction between Akt and its upstream activator MK2, which further mediate the recruitment of p38 in the complex leading to activation of MK2. These interactions promote activation of Akt through phosphorylation of S473 Akt as well as phosphorylation of Hsp27 at S82 [134–136]. Phosphorylation of Hsp27 by MK2 (also found *in vitro* by Akt) leads to its dissociation from the signalling complex [134–136]. Active Akt inhibits apoptosis in multiple ways, both upstream and downstream of mitochondrial perturbation [289]. It acts as a transducer of many functions initiated by growth factor receptors that activate phosphatidylinositol 3-kinase (PI3-kinase), and is a critical factor in cancer [290]. It can lead to inhibition of caspase-9 activity, phosphorylation of pro-apoptotic Bcl-2 family members such as Bad, or regulation of transcription factors such as cAMP-responsive element-binding protein and NF-κB and members of the Forkhead family [289,290]. Activated Akt can bind Bax and phosphorylate it at S183 [289], which inhibits its

conformational change and incorporation into and oligomerization in mitochondrial membrane, triggering the mitochondrial apoptotic cascade [251]. αA-crystallin and αB-crystallin prevent UV-induced apoptosis by activating distinctly the AKT and RAF/MEK/ERK pathways respectively [291]. Hsp27 protects adenocarcinoma cells from UV-induced apoptosis by Akt and p21-dependent pathways of survival [292]. Hsp27 prevented the G1-S phase arrest of the cell cycle upon UV-irradiation. Silencing Hsp27 was found to enhance G1-S phase arrest, leading to cell death [292].

Thus, sHsps participate in various signaling pathways of apoptosis/survival at different stages. It is to be noted that most of the studies performed in this context have specific objective of looking at a particular sHsp with regard to specific apoptotic stimuli and specific signaling pathways. It also appears that various apoptotic stimuli such as chemical (e.g., anti-cancer drugs) inducer, oxidative stress, cytokines (inflammation), heat shock and UV radiation also elicit overlapping and parallel signaling cascades and many such stimuli also lead to mediation of ROS. It is known that heat shock can lead to oxidative stress; oxidative stress and inflammation can lead to local increase in temperature. It is therefore, reasonable to envisage that many of the sHsps described above such as αB-crystallin, Hsp27, Hsp22 and Hsp20, which are stress-inducible as well as ubiquitously present, have a concerted participation/role in various apoptotic or survival pathways at different stages to elicit robust stress tolerance and cell survival.

1.15. sHsps and autophagy

Autophagy is important in promoting cell survival in several stress conditions such as protein aggregate formation, nutrient and growth factor deprivation, ER stress and pathogen infection [293,294]. Defective autophagy is associated with neurodegeneration, lysosomal storage diseases, muscular dystrophies, cancers and Crohn's disease [293]. Hsp22 forms a complex with Bag3, a stimulator of macroautophagy, in cells [295]. Over-expressing Bag3 or Hsp22 stimulated the formation LC3-II, a key molecule involved in macroautophagy [295]. Over-expression of Hsp22 has been shown to prevent the aggregation of the mutated huntingtin, Htt43Q, a pathogenic form responsible for Huntington's disease [296]. The Hsp22-Bag3 complex (along with Hsp70) is important for the degradation of aggregates of poly Q repeat protein, Htt43Q by macroautophagy [295]. Bag3-binding to Hsp22 is mediated by two conserved IPV motifs located between the W- and the P-rich domains of Bag3, and deletion of these motifs suppresses the activity of Hsp22 in degradation of Htt43Q [297].

Up-regulation of Hsp22 and BAG3 specifically in astrocytes in the cerebral areas affected by neuronal damage and degeneration have been observed in the post-mortem brain tissue from patients of Alzheimer's disease, Parkinson's disease, Huntington's disease and spinocerebellar ataxia type 3 (SCA3), suggesting that their up-regulation enhances the ability of astrocytes to clear aggregated proteins released from neurons and cellular debris [298]. The mutants of human Hsp22 associated with peripheral neuropathy [K141E and K141N] were found to be significantly less efficient than wild-type Hsp22 in decreasing the aggregation of mutated ataxin 3 and P182L-Hsp27, indicating that impairment or loss of function of Hsp22 might accelerate the progression of protein folding/conformational diseases [299]. The possible significance of Hsp22-Bag3 complex in autophagy is that Hsp22 may recognize the misfolded/aggregated proteins and Bag3, at least in part through its P-rich domain, recruit and activate the macroautophagy machinery in close proximity to the Hsp22-bound substrates [300].

Bag3, through its conserved IPV motif, interacts with Hsp20 [297] and αB-crystallin [301] as well. Whether such interaction with these sHsps also promotes autophagy is still to be established. Interestingly, Bag3 also interacts with the cardiomyopathy causing R120GαB-crystallin and its overexpression suppresses the aggregation and toxicity of R120GαB-crystallin [301]. HspB7 is also shown to be a very potent suppressor of the aggregation and toxicity of polyQ proteins, and the mechanism of its action probably involves stimulation of autophagy [182].

1.16. Role of sHsps in protein degradation

There are various lines of evidence as discussed below showing the involvement of sHsps such as Hsp27 and αB-crystallin in proteasomal degradation of proteins primarily promoting poly-ubiquitylation. The role of these sHsps seems to be of an adaptor, binding both the unfolded substrate and being part of complexes involving poly-ubiquitylation. Hsp27 also binds to poly-ubiquitin and proteasome facilitating the degradation. Ubiquitylation of proteins occurs via a multistep enzymatic reaction in which the polypeptide ubiquitin is covalently attached to the ε-amino group of a lysine side chain of the substrates. Polyubiquitylation of proteins targets them for proteolytic degradation by 26S proteasome [see review 302].

Alexander disease is a fatal neurodegenerative disorder caused by heterozygous mutations of the intermediate filament protein, glial fibrillary acidic protein (GFAP) involving the formation of aggregates of the mutant protein with associated components of αB-crystallin, Hsp27, ubiquitin and proteasome [303]. Up-regulation of these sHsps occurs in mice carrying GFAP mutations. The drug ceftriaxone could halt progression and ameliorate some of the symptoms of an adult form of Alexander disease. It is shown to reduce the intracytoplasmic aggregates of mutant GFAP in a cellular model of Alexander disease. The underlying mechanisms involve up-regulation of Hsp27 and αB-crystallin, polyubiquitination and degradation and autophagy [303]. The oligomeric form of a mutant GFAP (R239C) inhibits proteasomes. αB-crystallin could reverse this inhibition and promote proteasomal degradation of the mutant protein [304].

αB-crystallin has an important role in degradation of cyclin D1. SCF E3 ubiquitin ligase, which is primarily responsible for cyclin D1 degradation, consists of the F-box protein, Fbx4, and αB-crystallin as the substrate adaptors [305,306]. D-type (1, 2 and 3) cyclins bind cyclin-dependent kinases 4 or 6 (cdk4/6) which catalyze the phosphorylation of Rb proteins and promote G1/S phase transition; cyclin D1 is frequently overexpressed in human cancer [306]. Inhibition of ubiquitin-dependent proteolysis of cyclin D1 is believed to be a primary mechanism of cyclin D1 overexpression in human tumors [306]. Thus, as the FBX4-αB-crystallin complex is involved in the substrate recognition of SCF E3 ligase, which may have a role in tumor suppression [306]. S19 and S45 of αB-crystallin are preferentially phosphorylated during the mitotic phase of the cell cycle [239]. The interaction of αB-crystallin with FBX4 was found to be increased by mimicking phosphorylation of αB-crystallin at both S19 and S45 (S19D/S45D) [239].

In addition to its well characterized role in transcriptional regulation, SUMO (small ubiquitin-like modifier) modification plays a role in ubiquitin-mediated protein degradation in both nuclear and cytoplasmic compartments [77, see reviews 307,308]. Hsp27 promotes the SUMOylation and degradation of the mutant F508del cystic fibrosis transmembrane conductance regulator (CFTR) through interaction with the SUMO E2 enzyme, Ubc9 [77]. Under certain stress conditions, Hsp27 promotes ubiquitylation and proteasomal degradation of p27Kip1, an inhibitor of cyclin-dependent kinase (Cdk), which favours cell cycle progression [309]. It appears that the outcome of the role of αB-crystallin in cyclinD1 degradation and the role of Hsp27 in degradation of the Cdk inhibitor are mutually opposite; while the former suppresses cell cycle progression, the latter promotes it.

Hsp27 over-expression in various cell types enhances the degradation of ubiquitinated proteins by the 26S proteasome in response to treatment of etoposide or tumor necrosis factor α (TNF-α) by binding to polyubiquitin chains and to the 26S proteasome *in vitro* and *in vivo* [310]. Hsp27 enhances proteasomal degradation of phosphorylated I-κBα, the inhibitor of the transcription factor, NFκB leading to its activation and thereby elicits anti-apoptotic effects [310]. Hsp22 has been shown to co-localize with proteasome and over-expression of Hsp22

in cardiomyocytes leads to increased proteasome activity and cell growth [311]. The mechanism of how Hsp22 expression and interaction leads to increased proteasome activity is not known. Thus, these sHsps play a role in regulation of cellular process by facilitating degradation of key regulatory factors. sHsps-mediated proteasomal degradation of partially unfolded, aggregation-prone proteins underlies one of the mechanisms of clearing unwanted and refolding-incompetent proteins.

1.17. Circulating sHsps: role in immune system

Elevated levels of Hsp27 and αB-crystallin have been found in the serum and cerebrospinal fluid of patients of multiple sclerosis [312, 313]. Plasma level of Hsp27 was significantly higher in patients with acute coronary syndrome than in the normal reference subjects [314]. However, plasma levels of Hsp27 were found to be decreased in atherosclerotic patients compared with healthy subjects [315]. Estrogen induces release of Hsp27 in to serum and is atheroprotective [316]. Hsp27 binds scavenger receptor-A, reduces cholesterol uptake in macrophages, and attenuates mediators of vascular inflammation [316]. A temporal increase in the level of Hsp27 in serum has been found following acute ischemic stroke [317]. Enhanced level of Hsp27 in serum has been found in the cases of hepatocellular carcinoma [318] and pancreatic cancer [319]. High Hsp27 levels in serum were found to be associated with better nerve function and fewer neuropathic signs in normal glucose tolerance, impaired glucose tolerance and type 2 diabetes [320]. The levels of Hsp27 and its antibody in serum appear to relate to the presence of cardiovascular complications in patients with glucose intolerance [321].

The reason for the elevated levels of sHsps in serum could be either tissue damage or by secretion. The lack of an obvious signal sequence in sHsps rules out their secretion by the conventional pathway for their presence in the extracellular space. In fact, inhibitors of the conventional Endoplasmic reticulum-Golgi secretory pathway such as brefeldin A or tunicamycin do not affect secretion of αB-crystallin [322]. Exosome-mediated secretion seems to be one of the ways by which sHsps are found in the circulation. Presence of Hsp27 has been demonstrated in exosomes and its levels increase upon heat stress [323]. αB-crystallin [322,324] has been shown to be secreted through exosomes. Disruption of lipid raft (important for exosome secretion) by cyclodextrin leads to inhibition of the release of αB-crystallin, supporting the exosome-mediated secretion of the sHsp [324]. Circulating Hsp20 (HspB6) was elevated in a transgenic mouse model in which there was cardiac-specific over-expression, compared with wild-type mice; moreover, culture medium harvested from Hsp20-over-expressing cardiomyocytes contained an increased amount of Hsp20, indicating secretion of Hsp20 from cardiomyocytes [325]. Hsp20 was shown to be secreted through exosomes [325].

The roles of circulating sHsps in health and disease are not completely understood. Their roles may include stress signalling, angiogenesis, cell migration and immune modulation/regulation. sHsps appear to play an immune-regulatory role by stimulating macrophages to suppress inflammation. Therefore sHsps can be considered as therapeutic agents for inflammatory disorders [326]. However, in the case of αB-crystallin, adaptive immune responses against the protein itself may subvert the protective nature of the innate immune response it triggers, and this appears to be unique for humans [326]. Local concentrations of αB-crystallin may determine the balance between protective innate responses and destructive adaptive responses that is responsible for the development of multiple sclerosis lesions [326]. αB-crystallin is the most abundant protein present in multiple sclerosis lesions and elevated levels of αB-crystallin are found in the plasma of patients with multiple sclerosis (MS) and mice with experimental autoimmune encephalomyelitis (EAE) as compared with plasma from controls [327–329]. αB-crystallin has been shown to be the major target of CD4+ T-cell immunity to the myelin sheath from multiple sclerosis brain and this seems to be specific for human [327].

αB-crystallin knock-out mice with EAE exhibited greater degrees of paralysis, more widespread inflammation, and increased infiltration of activated CD4+ lymphocytes and macrophages and greater levels of glial apoptosis than the corresponding wild type animals [330]. Interestingly, exogenous (intravenous) administration of αB-crystallin to these αB-crystallin knock-out animals strikingly decreased these symptoms [330].

Exogenous administration of αB-crystallin in animal models of stroke [331], ischemia-reperfusion injury in the eye [332] and the heart [333] and CNS injury [334] has been shown to exhibit therapeutic benefits. Inflammation is the common event in all these autoimmune, stroke and acute ischemic conditions, indicating a role for αB-crystallin during inflammation. α-Crystallin (comprising both αA- and αB-crystallin subunits) pretreatment effectively diminished systemic inflammation-induced expression of glial fibrillary acidic protein (GFAP) and NFκB in the neocortex, reversed elevated intracellular levels of calcium, acetylcholine esterase activity and depletion of glucose in a mouse model of silver nitrate-induced inflammation [335]. Administration of αB-crystallin to mice challenged with LPS rapidly and efficiently reduced the plasma concentration of an inflammatory cytokine IL-6, showing an anti-inflammatory role of αB-crystallin [336]. Splenocytes from exogenous αB-crystallin-treated (intraperitonial) mice after ischemic stroke when stimulated secrete more of anti-inflammatory cytokine (IL-10) and lesser pro-inflammatory cytokines (IL-2, IL-17, IFN-γ, IL-12p40, and IL-6) than the respective controls [331]. Such anti-inflammatory/immunomodulatory role of αB-crystallin could underlie one of the mechanisms for the observed protective/therapeutic outcome upon administration of αB-crystallin even 12 h after experimental stroke in mice which reduced both stroke volume and inflammatory cytokines associated with stroke pathology [331]. αB-crystallin appears to be better than even the tissue plasminogen activator, administration of which is the only available treatment for stroke currently and it has to be administered within 4.5 hr of the onset of symptoms due to stroke [331].

Administration of recombinant human αB-crystallin to mice after contusion injury in spinal cord results in improved loco motor skills, amelioration of secondary tissue damage [334]. The recombinant protein modulates inflammatory response in the injured spinal cord leading to increased infiltration of granulocytes and decreased recruitment of inflammatory macrophages [334]. Moreover, the beneficial improvements were also observed upon exogenous administration of αB-crystallin even 6 h after spinal cord injury [334].

It appears that αB-crystallin not only limits the secretion of pro-inflammatory cytokines and increases secretion of the anti-inflammatory cytokines, but also can bind to pro-inflammatory molecules, probably to sequester them and inhibit their inflammatory function. Temperature-dependent conformational changes and increased chaperone ability of the protein also appears to be important in its anti-inflammatory role. Immuno precipitation and mass spectral analysis showed a common set of approximately 70 ligands for αB-crystallin from plasma from patients with multiple sclerosis, rheumatoid arthritis, and amyloidosis and mice with EAE [336]. More than half of these targets constitute acute phase proteins or members of the complement or coagulation cascades [336]. Interestingly, of the 70 proteins whose concentration was enhanced in the αB-crystallin immunoprecipitate, 67 were temperature-sensitive (enrichment in the precipitate and the increase in their binding with temperatures at 23, 37, or 42 °C); the acute phase, complement, and coagulation proteins were highly represented in this set suggesting that specific interactions between the chaperone and the targets could predominate in the elevated temperatures at sites of inflammation [336]. Thus, αB-crystallin exhibits anti-inflammatory effects by temperature-dependent binding of pro-inflammatory proteins in plasma, which can in turn influence both the innate and the adaptive immune responses [336].

Similarly, Hsp27 also plays an anti-inflammatory role. Exogenous addition of Hsp27 to a monocyte culture induces IL-10 via activation of p38 signaling independent of TNF-α activation, indicating an anti-

inflammatory role for Hsp27 [337]. However, treating macrophages with Hsp27 leads to the degradation of an inhibitor of NF-κB, IκBα, nuclear translocation of the NF-κB p65 subunit and increased NF-κB transcriptional activity, resulting in increased expression of the pro-inflammatory factors, IL-1β, and TNF-α as well as the anti-inflammatory factors IL-10 and GM-CSF [338]. Exogenous treatment of monocytes with Hsp27 inhibited the differentiation of monocytes to the potent antigen presenting, matured dendritic cells [339], indicating that extracellular and/or exogenous Hsp27 could have immuno-suppressive role as well.

As mentioned earlier, specific T-cell-mediated immune response raised by αB-crystallin overwhelms the beneficial anti-inflammatory response it triggers in the specific cases of multiple sclerosis [327]. Though exogenous treatment with αB-crystallin can have therapeutic potential as demonstrated in animal model [330], it can still sustain or even promote the T-cell-mediated adaptive immune response. It would be interesting to address whether the anti-inflammatory effect brought out by administration of Hsp27, instead, would have a better outcome in symptomatic relief and frequency of attack in animal models. It would also be important to address the relative anti-inflammatory potency among these sHsps for developing sHsps as therapeutic molecules for inflammatory diseases.

Besides the involvement of intracellular sHsps, their presence in extracellular circulation (e.g., αB-crystallin, Hsp27 and Hsp20) also promotes angiogenesis. Treating endothelial cells with recombinant Hsp27 leads to internalization of Hsp27 to endosomes through toll like receptor TLR3 and induces NF-κB activation, secretion of VEGF, increased cell migration and angiogenesis [340]. Circulating Hsp20 was increased in a transgenic mouse model with cardiac-specific overexpression of Hsp20, and capillary density was significantly enhanced in hearts overexpressing Hsp20, compared to that in wild type mouse [325].Treating human umbilical vascular endothelial cells with Hsp20 promotes cell proliferation, migration and tube formation via activation of VEGFR2, indicating that Hsp20 serves as a novel cardiokine in regulating myocardial angiogenesis [325].Thus, the circulating sHsps also play important physiological and pathophysiological functions. The overall effect appears to be beneficial in nature and such beneficial effects could be augmented by exogenous administration.

1.18. Circulating sHsps: role in anti-platelet aggregation

sHsps, particularly, αB-crystallin, Hsp27 and Hsp20 are present in high concentrations in vascular smooth muscle cells [see review 225]. The level of Hsp20 in circulation is high in patients with vascular disease and in cardiomyopathic hamsters [341,342]. Hsp20 inhibits platelet aggregation *in vitro* and *ex vivo* [341,343]. On the other hand, Hsp27 does not inhibit platelet aggregation [343]. Hsp20 inhibits thrombin-induced calcium influx, which is correlated with the suppression of thrombin-induced platelet aggregation by Hsp20 [341]. Hsp20 binds to platelets and reduces thrombin-induced phosphoinositide hydrolysis by phospholipase C, indicating that extracellular Hsp20 can cause intracellular signaling events [342]. Recombinant cell-permeable Hsp20 (TAT-HSP20) has been shown to inhibit vascular smooth muscle contraction (vasospasam) and platelet aggregation (thrombosis) in a rabbit model [344].

The concentration of αB-crystallin in plasma of cardiomyopathic hamster increases approximately 14-fold compared to normal animal [345]. αB-crystallin binds to platelets, inhibits thrombin-induced calcium influx and exhibits anti-platelet aggregation [345]. Interestingly, a peptide (^{9}WIRRPFFPF^{17}) derived from αB-crystallin inhibits platelet aggregation induced by thrombin, TRAP (agonist of protease activated receptor-1) and botrocetin, ristocetin (stimulator of the platelet glycoprotein Ib/V/IX-von Willebrand factor axis), but not collagen and ADP (protease-activated receptor-4 agonist) [346]. The peptide was also found to exhibit significant antithrombotic effect without marked bleeding *in vivo* [346]. Extracellular treatment with αB-crystallin affects the intracellular signal transduction event during platelet activation. Treating platelets with αB-crystallin attenuates the adenosine diphosphate (ADP)-induced phosphorylation of p44/p42 mitogen-activated protein kinase (MAPK) and p38 MAPK, and hence ADP-stimulated phosphorylation of Hsp27 and secretion of platelet granules (which contains platelet-derived growth factor (PDGF)-AB, serotonin and soluble CD40 ligand (sCD40L) [347].

Atherosclerosis causes platelet adhesion, activation, and aggregation at sites of vascular endothelial disruption, which are critical events in arterial thrombus formation. Anti-platelet therapy is widely used for prevention of ischemic cardiac complications in patients with acute coronary syndrome [348]. Oral anti-platelet agents including ticagrelor, prasugrel, or clopidogrel in combination with aspirin are used with their own advantages and complications [348]. Activation and aggregation of platelets not only impact coronary thrombus but are major contributors to microcirculatory dysfunction and vascular inflammation in acute coronary syndromes. Therefore, efforts to inhibit platelet activation and aggregation, including anti-platelet therapy, are paramount to the management of acute coronary syndromes [349]. It is intriguing and important to get further understanding on the effect of sHsps in both platelet activation and aggregation and to evaluate the relative efficiencies of sHsps and their peptides among themselves and as compared to conventional drugs used for anti-platelet therapy. It would be also be interesting to investigate whether these sHsps and their peptides either alone or in combination with conventional drugs further improve the treatment outcome of anti-platelet therapy.

1.19. sHsps in fertilization and development

The sHsp ODF1/HspB10 is essential for male fertility in mice. HspB10 is not only required for the correct arrangement of mitochondrial sheath and outer dense fibers of sperms, but is also needed for the rigid junction of the sperm head and tail [350]. The expression of murine Hsp25 mRNA was increased in the testis with onset of spermatogenesis and progression to adulthood [351]. The abundance of Hsp25 mRNA was also found to vary in the seminiferous tubules that were at different stages of spermatogenesis [351]. Patients with polycystic ovary syndrome (PCOS) are typically characterized by increased numbers of oocytes which are often of poor quality, leading to lower fertilization, cleavage and implantation rates, and a higher miscarriage rate [352]. Hsp27 has been found to be down-regulated in ovarian tissue derived from women with PCOS [353]. Over-expression of Hsp27 in oocytes derived from PCOS patients leads to inhibition of oocyte maturation, but improves embryonic developmental potential by down-regulating oocyte-secreted factors, BMP15 and GDF9, and the apoptotic-related regulators, Caspase 3, 8 and 9 [353]. Downregulation of Hsp25 improved the maturation of mouse oocytes but increased early stage of apoptosis through the activation of extrinsic, caspase 8-mediated pathway [354]. Though the exact mechanism of these observations still needs to be explored, it appears that Hsp27/Hsp25 critically regulates oocyte maturation and its developmental potential through regulation of apoptosis as one of the mechanisms. Perinatal and developmental expression pattern of sHsps in several tissues such as lens, brain, heart, liver, kidney, lung, skeletal muscle, stomach, and colon in piglets showed that each tissue had a unique sHsp expression pattern that varied during development [355]. The precise reasons for this distinct temporal and tissue-dependent expression pattern in each tissue are not well understood, but are suggestive of roles of each of these sHsps in the different developmental stages.

1.20. sHsps and differentiation

The levels of Hsp27 and αB-crystallin, have been shown to increase 3-fold and 10-fold respectively during the differentiation of mouse myoblasts to myotubes though the levels of Hsp70 did not change significantly [10]. A study from our laboratory has shown that Hsp27 and αB-crystallin localize to the cytoplasm of myoblasts under normal

conditions, but translocate to the nucleus under conditions of stress, co-localizing with the laminA/C nuclear speckles [237]. On the other hand, both Hsp27 and αB-crystallin localize only to the cytoplasm in myotubes under normal as well as heat stress conditions, indicating a stage-specific role of these sHsps during differentiation [237]. One of the mechanisms by which sHsps regulate/aid differentiation process is to prevent the differentiation-induced apoptosis. Differentiation requires activation of some of the caspases; at the same time, the activation process needs to be controlled or regulated to avoid apoptosis, for which the sHsps are probably recruited. αB-crystallin prevents apoptosis induced by TNF-α (one of the myogenic regulators) by activating NFκ B [279]. Expression of Hsp22 increased during neuronal differentiation, particularly in the later stages, and silencing Hsp22 was shown to affect neuronal differentiation [356]. Hsp22 increases cell survival by enhancing phosphorylation of Akt, thereby promoting neuronal differentiation [356]. Interestingly, even the truncated form of Hsp22 comprising its ACD was sufficient to promote cell survival and neuronal differentiation [356].

In addition to this "apoptotic check" mechanism, sHsps can also modulate the activities of myogenic factors, thereby regulating the differentiation processes. Analysis of the promoter region of αB-crystallin indicated the presence of potential binding sites for muscle regulatory factors MyoD1, myogenin, Myf-5, and MRF4 in the muscle-preferred enhancer region (-426 to -257) [357]. A study from our laboratory has shown that αB-crystallin modulates MyoD activity by its combined effect on degradation and synthesis of MyoD, thereby delaying muscle differentiation [358].

Hsp27 is up-regulated during the Ca^{2+}-induced differentiation of keratinocytes [359]. The level of Hsp25 (the mouse ortholog of Hsp27) increases with the distance of keratinocytes from the basal layer, in parallel with the extent of keratinization during epidermis differentiation. Hsp25 is involved in two steps of PAM212 keratinocyte differentiation – (i) A transient hyperphosphorylation of Hsp25, shortly after the induction of differentiation seems to be essential for the expression of differentiation markers and (ii) Hsp25 is later organized into characteristic aggregates involved in the dynamics of keratin filament networks [360]. Whether sHsps have a regulatory role in differentiation processes in general including stem cell differentiation and the mechanistic details of the roles of individual sHsps in a given differentiation process need further investigations.

1.21. sHsps in health and disease

The functions of sHsps (e.g, αB-crystallin, Hsp27, Hsp22 and Hsp20) have both beneficial and deleterious outcomes. The sHsps have beneficial outcomes in neuroprotection, function of heart and its protection against ischemia, and in myogenesis and muscle homeostasis. On the other hand, their function has deleterious outcomes in cancer and the development of drug resistance [361].

Hsp27 has powerful neuroprotective effects and over-expression of Hsp27 in transgenic animals confers neuroprotection as well as protects against cardiac ischemia [see reviews 362,363]. Hsp27 shows constitutive expression in some areas of the mammalian retina and is up-regulated in response to ischemia and oxidative stress, traumatic nerve injury, elevated intraocular pressure and glaucoma [see review 225]. Over-expression of Hsp27 confers long-lasting neuroprotection against ischemic brain injury via inhibition of ASK1 kinase signalling [271]. Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disorder, characterized by progressive motor neuron degeneration and muscle paralysis. Over-expression of Hsp27 in a mouse model of ALS (G93ASOD1) showed delayed decline in motor strength, an improvement in the number of functional motor units and increased survival of spinal motor neurons [365]. Several mechanisms account for the cytoprotective actions of Hsp27, including its role as a molecular chaperone, a stabilizer of the cytoskeleton, and a regulator of apoptosis [362,364].

Huntington's disease (HD) is an inherited neurodegenerative disorder caused by an expansion of glutamine repeats in the huntingtin (htt) protein due to abnormal protein folding and the accumulation of mutated htt. Hsp104 and Hsp27 rescue striatal dysfunction in primary neuronal cultures and HD rat models [366]. Formation of fibrillar aggregates of proteins containing polyglutamine (polyQ) expansion leads to neuronal cell death in nine diseases, including HD and spinocerebellar ataxias (SCAs) [367]. Proteins such as ataxin-1 (in SCA1), ataxin-3 (in SCA3), and huntingtin (in HD) form fibrils by a multidomain misfolding mechanism involving aggregation of not only the polyQ regions but other aggregation-prone regions as well [368,369]. For example, aggregation of ataxin-3 involves the first stage of aggregation of the globular N-terminal Josephin domain followed by the self-association of expanded polyQ segments [368]. αB-crystallin suppressed SCA3 toxicity in a Drosophila model [370]. Suppression was more pronounced when αB-crystallin was co-expressed with full-length ataxin-3 than when co-expressed with a C-terminal fragment not containing the Josephin domain [370]. αB-crystallin did not prevent the fibril formation of a polyQ protein (SpAcQ52) that forms fibrillar aggregates by a mechanism involving only the polyQ region [367]. However, αB-crystallin inhibited the initial Josephin domain-dependent stage of aggregation of ataxin-3 harboring a pathological length polyQ tract [at3(Q64)] and a truncated variant comprising only the Josephin domain [367]. Interestingly, Hsp22, but not other sHsps such as Hsp27 and αB-crystallin, prevents Htt43Q protein aggregation and perinuclear accumulation of inclusions of the SDS-insoluble aggregates in the cells, suggesting that Hsp22 functions as a molecular chaperone, maintaining Htt43Q in a soluble state competent for rapid degradation [296]. Properties other than chaperone activity of sHsps could also offer neuronal protection, as sHsp over-expression decreases neuronal toxicity in HD by suppression of ROS [371] and stimulation of autophagy [372].

Antiapoptotic and cytoprotective properties of some sHsps are used by cancer cells to their advantage. Hsp27 and αB-crystallin have been considered as oncoproteins [373]. Their function also bears deleterious consequences in angiogenesis and cancer cell metastasis. αB-crystallin expression is associated with distant metastases formation in head and neck squamous cell carcinoma patients [374]. αB-crystallin was found to be specifically induced during tubular morphogenesis of endothelial cells and knockdown of αB-crystallin expression led to attenuated tubular morphogenesis [375]. αB-crystallin and VEGF-A (essential factor in angiogenesis) are colocalized in the endoplasmic reticulum in RPE cells under chemical hypoxia [376]. αB-crystallin binds to VEGF-A in cultured retinal pigment epithelial (RPE) cells. αB-crystallin(−/−) RPE showed low VEGF-A secretion compared with wild-type cells, indicating that αB-crystallin mediates folding and secretion of VEGF [376]. Extracellular addition of Hsp27 leads to internalization of Hsp27 to endosomes through toll like receptor TLR3 and induces NF-κB activation, secretion of VEGF, increased cell migration and angiogenesis [340]. Down-regulation of Hsp27 leads to reduced endothelial cell proliferation and decreased secretion of VEGF-A, VEGF-C, and basic fibroblast growth factor; conversely, over-expression of Hsp27 in non-angiogenic cells leads to aggressive tumor growth *in vivo*, indicating involvement of Hsp27 in tumor angiogenesis [377]. High level expression of Hsp27 correlates with the aggressiveness of several primary tumors and bone-metastasis potential of breast cancer cells [378].Treatment of human umbilical vein endothelial cells with recombinant human Hsp20 (HspB6) promotes proliferation, migration and tube formation involving interaction between Hsp20 and VEGFR2 [325]. Hsp27 inhibits maturation of dendritic cells, and therefore seems to be immune-suppressive [339]. Dendritic cells are potent antigen presenting cells that activate B and T lymphocytes as well as natural killer cells [379]. Therefore Hsp27-induced inhibition of dendritic cell maturation can facilitate the evasion of immune response by cancer cells. Thus, targeting some of these sHsps either to decrease their expression or to inhibit their function would be a promising strategy for cancer therapy.

Another case of adverse outcome due to the high expression of sHsps is the demonstrated involvement of αB-crystallin in idiopathic pulmonary fibrosis [380]. Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by the proliferation of myofibroblasts and the accumulation of extracellular-matrix in the lungs, mediated by the pro-fibrotic cytokine, TGF-β1 [381]. αB-crystallin is highly expressed in fibrotic lung tissue from IPF patients as well as from rodent models of pulmonary fibrosis [380]. Moreover, αB-crystallin-deficient mice are protected from fibrosis induced by bleomycin or transient adenoviral-mediated over-expression of TGF-β1 or of the pro-inflammatory cytokine IL-1β (which also increases TGF-β1) [380]. αB-crystallin increases the nuclear localization of Smad4, enhancing the TGF-β1/Smad pathway and the consequent activation of TGF-β1 down-stream genes in primary epithelial cells and fibroblasts *in vitro*. αB-crystallin over-expression inhibits mono-ubiquitination of Smad4 (probably by disrupting Smad4 interaction with E3-ubiquitin ligase, TIF1γ) which disfavors otherwise mono-ubuquitination-favoured nuclear export of Smad4, thus increasing nuclear localization and activity of Smad4 complex [380]. Thus, considering both beneficial and adverse outcomes of sHsps expression, conditional and targeted functional modulation of the activities of sHsps is the most important and the most challenging aspect that needs to be addressed for disease managment.

1.22. "Dynamic partitioning hypothesis" for promiscuous interactions and pleotropic functions of sHsps

As many as 72 and 78 different targets have been known for Hsp27 and αB-crystallin respectively [see review 143]. Interestingly, only about 10% of these targets, mostly either cytoskeletal elements or aggregation-prone or amyloidogenic proteins/peptides (inferred from ref. 143), are common to both Hsp27 and αB-crystallin, exhibit both shared and exclusive functions. Studies probing the mechanistic details using various model proteins/peptides yielded several putative substrate-binding regions (sites) spanning almost the entire length of proteins depending on the substrates used [61,103,120,121,382–385]. Proteomic investigations indicated as high as ~300 unfolding targets (under heat stress) of HeLa cell lysate for αB-crystallin [72]. Hsp27 was found to be widely distributed from ~20 kDa to ~900 kDa fractions of the cell-extract upon gel-filtration chromatography [141, see reviews 143,218], suggesting that it exists in such diverse multimeric populations or its species are associated with diverse sets of proteins and hence elutes in different fractions with widely distributed molecular masses. The diverse substrate-interacting regions observed for mammalian sHsps also seem to be applicable to plant sHsps. Model substrates do not bind a discrete surface but make contacts throughout the pea PsHsp 18.1 [386]. Probing dynamics of complexes formed between the oligomeric pea-sHSP18.1 and the client luciferase by mass spectrometry strategies reveals over 300 different stoichiometries of interaction [387].

Thus, the interacting partners of sHsps are too many, as well as involved in diverse cellular processes, to invoke highly specific, well-defined interactions for sHsps with their clients to form specific complexes. Thus, the interactions of sHsps with their targets seem to be pseudo specific (or broadly specific) in nature. Nevertheless, these interactions of sHsps have important physiological and pathophysiological consequences. Therefore, understanding the mechanism(s) involved in the promiscuous substrate interactions and pleotropic functions of sHsps is important.

We propose a dynamic partitioning hypothesis for the observed promiscuous interaction of sHsps with clients, taking into consideration the emerging importance of the dynamic assembly, structural plasticity and flexibility of the N- and C-terminal regions of sHsps (e.g., αB-crystallin). We have earlier shown that the chaperone activity of αA- and αB-crystallin towards citrate synthase involves: (i) a relatively weak and reversible interaction with early unfolding intermediates that prevents partitioning of these intermediates to late-unfolding intermediates leading to reactivation, and (ii) an irreversible, stable interaction with the aggregation-prone late unfolding intermediate, keeping the complex in solution [25]. Two modes of interactions involving low and high affinities and different stoichiometries determined by the dynamic population of folding intermediates have been proposed for the mechanisms of chaperone activities of sHsps such as αA-, αB-crystallin and Hsp27 with model substrates by another laboratory [33–36]. The observed intrinsic plasticity of conformation and dynamic nature of N- and C-termini of some sHsps [72,106,112] and subunit exchange process potentially leading to polydispersity [see review 158] indicate a versatile mode of interactive-potential of sHsps. This versatility can further be modulated by phosphorylation and hetero-oligomer formation between sHsps.

Fig. 6 schematically depicts the dynamic partitioning hypothesis. We envisage that the dynamic ensembles of sHsp species partition themselves with yet another dynamic process involving substrates, mediated by two types of interactions: (i) reversible interactions with various targets setting up an equilibrium (Type 1 interactions) and (ii) relatively stronger and less reversible interactions (Type 2 interactions). The degree of Type I interactions depends on the nature of the equilibrium species populated among native or native-like species to early unfolding intermediates of the target proteins. Some pseudo-specific regions on the targets become temporally accessible to sHsps (the accessibility may be determined by conditional phosphorylation, stress conditions or loss or gain of interaction with other partners of the given target). sHsps form dynamic fractional populations of reversible complexes with their targets (with sufficient residence time of the complex to be able to be detected by various biophysical and cell biological techniques). Stimuli (stress or external agents) lead to temporal variation in both the number and the accessibility of pseudo-specific sites of targets which would affect the partitioning of sHsps towards those particular targets. In addition, binding-induced stabilization or steric constraints upon their interaction with sHsps would also determine the apparent, gross selectivity towards target proteins exhibited by individual sHsps. Knocking in or knocking out of sHsps, would, thus, lead to alteration of this dynamic equilibrium and perceptible phenotypes (or detectable outcome).

Type I interactions between sHsps and their targets help maintain the cellular homeostasis under permissible and early stress conditions, which would give the cellular system kinetic advantage to cope up with perturbations and help elicit further protective mechanisms. Some examples of type I interactions are as follows. Interactions of αB-crystallin or Hsp27 tend to partition Bax in its inactive form. Interaction of some sHsps with cytochrome c would lead to temporal depletion of cytochrome c, retarding the down-stream processes of apoptosis. A small fraction of the population of the cytoskeleton is bound by some sHsps. Under stimuli which elicit a particular process, for example, apoptosis-inducing conditions, sHsps partition more to the complexes with proapoptotic factors (Bax, cytochrome c etc.). Similarly a fraction of sHsps species may also simultaneously be engaged with a fraction of anti-apoptotic factors or pro-survival machinery. Type 1 interactions with some clients which are in equilibrium with early unfolding intermediate states would also prevent further partitioning of these states to the relatively more unfolded aggregation-prone intermediate states of the protein as proposed earlier [25]. In some cases, type 1 interactions of sHsps might increase the activity of the targets. For example, phosphorylated αB-crystallin interacts with IKKβ and increases its kinase activity, subsequently leading to NFκB activation [279]. Phosphorylated Hsp27 interacts with Glucose 6 Phosphate Dehydrogenase, increasing its activity [388]. HspB2 interacts with DMPK, increasing its kinase activity [389].

Though we are far from a comprehensive understanding on the pseudo-specific regions of targets recognized by sHsps, at least the "IXI/V"-like motif on targets may be attributed to be one of the pseudo-specific regions. For example, the IPV motif of Bag3 can interact

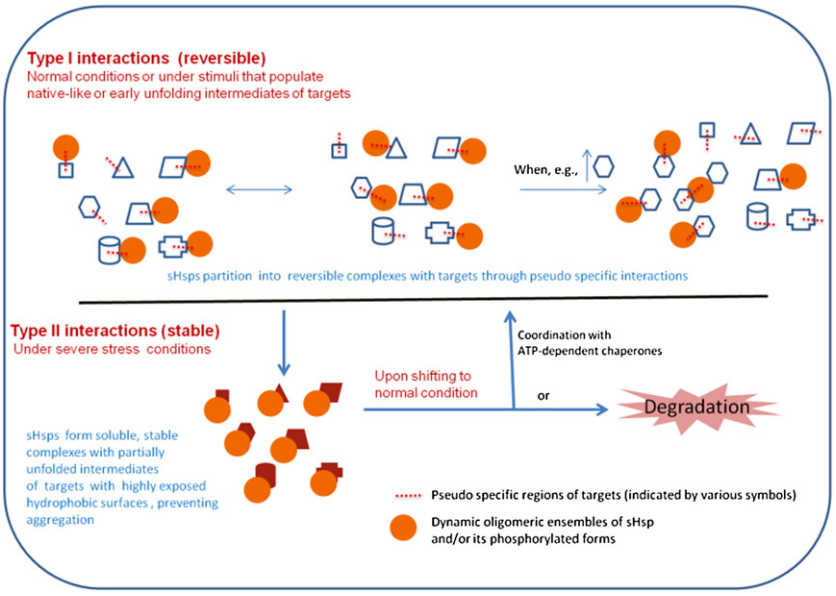

Fig. 6. Schematic representation of the "Dynamic partitioning hypothesis" for the promiscuous interactions and pleotropic functions of sHsps.

with the β4-β8 groove of ACD of αB-crystallin/Hsp20 [297,301]. Thus, self versus client IXI/V motif can compete for the same interacting site. This interaction with target is also likely to be dynamic in nature as the self-IXI/V motif CTE interaction with subunits of αB-crystallin is shown to be dynamic and is temperature-dependent [127,128]. More types of such putative sHsp-interacting regions on clients may make dynamic, pseudospecific contacts with sHsps, mediating type 1 interactions.

(ii) The Type II interactions of sHsps with aggregation-prone unfolding intermediates of the target proteins (under severe stress) are relatively stronger and less reversible. Temperature-dependent perturbations of sHsps at the tertiary structure with increased exposure of hydrophobic surfaces (for example eye lens α-crystallin [21]), flexibility of N- and C- terminal regions [36,74,106,112] and the increased dynamic exchange-mediated alteration of quaternary structure [158] would enable the sHsps to accommodate themselves on the exposed hydrophobic contour of the target protein to form relatively more stable complexes. Like octopus arms, the flexible NTD, can warp around/mask the hydrophobic surface and stabilize the complex of some target proteins. The NTD may also interact and stabilize target proteins which are anchored to the ACD (β3, β8 and β9 regions appear to be common for many target proteins [61]). Thus, depending on the accessible surface contour of the target protein, the interacting/docking residues/regions of the sHsps would differ, explaining the various experimentally observed interacting sites spanning the NTD and the core ACD of sHsps [61,103,120,121,382–385]. Such relatively more stable complex formed under conditions of stress could either be refolded or be targeted for degradation as discussed in earlier sections.

Though the "Dynamic partitioning hypothesis" is built on several experimental results as discussed earlier, a comprehensive investigation of distinct as well as common intra- and extra-cellular targets ("interactome") of sHsps would be important for further supporting the hypothesis. Moreover, identification of potential regions of the target recognized by sHsps, under experimental conditions, which preserve native or native-like species of proteome as well as under stress conditions (eg., heat or oxidative) which create pool of partially unfolded/denatured targets, would also be important.

A simplified analogy can be drawn between the sHsps and their client proteins and a doctor and his clients. The "Doctor" (sHsps) carries out general check-ups (type I interactions) and takes care of the well-being (cellular homeostasis) of his "clients" (substrate proteins) to prevent them from being "patients" (aggregation-prone states). The "Doctor" admits (type II interaction) the "patients" depending on the nature of the" illness" (degree and nature of unfolding) and treats alone or together with other "Specialists" (ATP-dependent chaperones) to send them back "Home" (native state), failing which, he sends them to the "Mortuary" (degradation). When the "Doctors" become "Patients" (point mutations of sHsps), the number of working "Doctors" decreases (recessive phenotype) as well as the "Doctor patients" take away (dominant negative phenotype) "Doctors for patients" for their own treatment and the system suffers (myopathies, neuropathies). What mediates (pseudo-specific regions) the apparent common and distinct clients' interactions with the "Doctors" is a puzzle to work on. In times of crises such as "epidemics" (stroke, ischemia or other acute diseases, neurodegeneration etc.,), the "in-house doctors" (endogenous sHsps) are insufficient, but the additional "guest doctors" (administration of recombinant sHsps) of the rescue team can carry out a potent rescue operation. By the very nature of goodness, the "Doctors" also treat and save "Bad people for the society" (cancer and pathogenic angiogenesis). How to make the "Doctors" discriminatory (targeted and conditional inhibition of sHsps) is a serious "administrative problem" to solve.

1.23. General perspective

As highlighted in this review, major information on pleiotropic functions of sHsps and their promiscuous interactions with several targets is with respect to the well studied sHsps, αB-crystallin and Hsp27, and to some extent with respect to Hsp20 and Hsp22. A major question that arises in this respect is: what are the common and distinct targets of these sHsps? Is it possible to dissect out information on individual and collective contributions of the NTD, the ACD and the CTE of a given sHsp towards its different targets? The sHsps mentioned above seem to have broad tissue distribution (Class 1 sHsps). The role of other sHsps that have restricted tissue distribution (Class 2 sHsps) in cellular function(s) is rather less understood. Some of the questions which need further investigations for a complete understanding on the subject of human/mammalian sHsps in health and disease conditions are as follows: What are the structural and functional differences between ubiquitous and tissue-restricted sHsps? What is the functional significance of muscle tissue having as many as six sHsps? It appears that the expression of different sHsps is temporally regulated during myogenic differentiation, and yet they are all present significantly in the differentiated muscle tissues [213]. Why their expressions are temporally regulated?

What is the biological significance of this temporal regulation? Answers to these questions would allow developing strategies for functional targeting of individual or a set of sHsps.

Expression levels of αA- and αB-crystallin are altered during the phases of cell cycle of lens epithelial cells and knock down or knock in of these crystallins affects cell cycle progression [390,391]. As discussed earlier (sHsps in protein degradation section), αB-crystallin and Hsp27 also influence cell cycle by involving in degradation of some of the factors such as cyclin D1 and p27Kip1, an inhibitor of cyclin-dependent kinase respectively. Considering the involvement of sHsps, especially Hsp27 and αB-crystallin in cancer, further understanding on the mechanistic aspects and significance of sHsps in cell cycle process is important.

As mentioned earlier, sHsps exhibit inter-sHsp interactions *in vitro* through subunit exchange process. Though hetero-species of sHsps have been isolated from cells/tissues [213], there is no clear *in vivo* demonstration as yet that these species are formed by subunit exchange process (between two pre-existing oligomers of sHsps). Crowding environment and heterogeneous visocity variations due to semi-ordering of molecules in a cellular environment can potentially affect subunit exchange processes. It is not clear whether formation of hetero-sHsp complexes is due to random stochastic events (because of their inherent potential to interact among themselves), or for purposes under the influence of certain stimuli. What is the significance of such hetero-sHsp complex formation? Is it possible to provide a structural basis for apparent selectivity among sHsps to form hetero-sHsp complexes? How does hetero-sHsp complex formation affect the dynamics and oligomeric size of the resultant complex? Whether hetero-sHsp complexes exhibit dual functions of the individual sHsps or acquire new functional surfaces would be an interesting aspect for future studies. It is not yet clear whether hetero-oligomeric formation between sHsps is spontaneous (as observed *in vitro*) or determined by as yet unknown condition(s) *in vivo*.

Though there have been indications that phosphorylation of αB-crystallin and Hsp27 affects oligomeric size, subunit exchange and probably plays a role in client-discrimination, further understanding with respect to other sHsps and their influence on hetero-sHsp interactions is important. As the point mutation in αB-crystallin, R120G, leads to hyper-phosphorylation [223,392], a detailed investigation of the role of this hyperphosphorylation in disease is required. Further investigation is needed to find out whether the phenomenon of hyper-phosphorylation is common to other mutations in sHsps. Whether modulation of phosphorylation would serve as a possible therapeutic intervention in mutation-linked myopathies and neuropathies is a possible direction for future investigations. Interaction of metal ions such as Cu^{2+} with αB-crystallin and Hsp27 is an emerging aspect. Is this a general property of sHsps? What are the metal ion(s)-mediated structural and functional aspects/changes of sHsps need to be addressed.

Some of the sHsps such as Hsp27, αB-crystallin and Hsp20 have been found in circulation and have been shown to be secreted through exosomes [322–325]. As discussed in one of the sections, αB-crystallin has been shown to have anti-inflammatory property by immune cell modulation or by binding to pro-inflammatory molecules, and external administration of the protein seems to have therapeutic potential. Do Hsp27 and Hsp20 also exhibit similar anti-inflammatory role and provide protection/therapeutic benefits upon external administration? If so how do these proteins compare in their potentials? It is possible to amplify the protective role of sHsps by external administration of recombinant sHsps/or their peptides for therapeutic benefits. Can we engineer sHsps possessing highly potent molecular chaperone activity as well as potent anti-inflammatory and anti-thrombotic activity to develop as a general drug or adjuvant drug for various ailments such as neurodegenerative diseases, heart-related diseases, stroke and autoimmune diseases like multiple sclerosis?

sHsps are also likely to play an important role in stem cell biology. There seems to be specific signature of expression of Hsps including some sHsps in embryonic, mesenchymal and neural stem cells and the levels of specific sHsps are altered during their differentiation [see review 393]. However, the significance of the temporal expression of sHsps in stem cells is not understood. As stem cells are increasingly being used for therapeutic purposes, exploring role of sHsps in maintaining stemness and in the process of differentiation will have potential applications.

Up- or down-regulation of small heat shock proteins could have beneficial outcomes under different conditions. The up-regulation of sHsps has been shown to provide beneficial effects in cardiac ischemia and in neurodegenerative disorders such as amyotrphic lateral sclerosis. On the other hand, down-regulation of sHsps could provide a beneficial outcome in cancer and age-related macular degeneration. Small molecules that can either up-regulate or down-regulate the heat shock response in general are known. Small molecules such as arimoclomol and bimoclomol are known to up-regulate the heat shock response, whereas certain small molecules such as quercetin have been shown to down-regulate the heat shock response in general [see review [394]. Quercetin inhibits heat shock factor-1 (HSF-1) expression and the binding of HSF-1 to the heat shock element [395]. Thus, temporal administration or targeted delivery of quercetin to cancer cells might show promising augmentation to chemotherapy. Besides the general heat shock response inhibitors, developing specific functional inhibitors of the sHsps (particularly Hsp27 and αB-crystallin) would be important. Peptide aptamers have been developed that specifically interact with Hsp27, and their expression in cell culture perturbed the dimerization and oligomerization of Hsp27, negatively regulated the anti-apoptotic and cytoprotective activities of Hsp27 and strongly reduced tumor development in a xenograft mouse model through cell cycle arrest [396]. These peptide aptamers interact with small oligomers of Hsp27, affect phosphorylation of Hsp27 and redistribution of larger oligomers with decreased S78 phosphorylation [94]. A recent study has used the crystal structure of the ACD of αB-crystallin as a basis for molecular docking to design a molecule, NCI-41356, that can block interaction between αB-crystallin and VEGF-165. Intraperitoneal injection of this molecule in an *in vivo* human breast cancer xenograft model resulted in inhibition of tumor growth and development of vasculature [397]. Molecules that selectively bind the NTD and the ACD of sHsps can be developed to inhibit interactions with set of their clients. Considering the potent anti-apoptotic and angiogenic properties of αB-crystallin and Hsp27, their over-expression especially in the metastatic tumor cells, and its correlation with drug-resistance to chemotherapy, developing a dual inhibition strategy either by common inhibitor(s) or respective inhibitors as an adjuvant therapy might give promising outcome of chemotherapy even at the advanced stages of tumors. More work towards developing specific small molecule inhibitors of sHsps which can easily be delivered is required. Since sHsps have pleotropic functions, general inhibition of their function may have side-effects. Therefore, targeted delivery systems of their putative inhibitors need to be parallelly developed for sHsp-based therapy for human diseases such as cancer and age-related macular degeneration (which involve pathological angiogenesis). Thus, conditional and targeted modulation of the pleiotropic functions of sHsps would emerge as a future direction of this area.

Statement of conflict of interest

Authors claim no conflict of interest.

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