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α B-crystallin: Portrait of a malignant chaperone as a cancer therapeutic target

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Abstract

α B-crystallin is a widely expressed member of the small heat shock protein family that protects cells from stress by its dual function as a molecular chaperone to preserve proteostasis and as a cell death antagonist that negatively regulates components of the conserved apoptotic cell death machinery. Deregulated expression of α B-crystallin occurs in a broad array of solid tumors and has been linked to tumor progression and poor clinical outcomes. This review will focus on new insights into the molecular mechanisms by which oncogenes, oxidative stress, matrix detachment and other tumor microenvironmental stressors deregulate α B-crystallin expression. We will also review accumulating evidence pointing to an essential role for α B-crystallin in the multi-step metastatic cascade whereby tumor cells colonize distant organs by circumventing a multitude of barriers to cell migration and survival. Finally, we will evaluate emerging strategies to therapeutically target α B-crystallin and/or interacting proteins to selectively activate apoptosis and/or derail the metastatic cascade in an effort to improve outcomes for patients with metastatic disease.

Keywords

Molecular chaperone; Stress response; Apoptosis; Cancer; Metastasis; Drug target

1. Introduction

Cancer cells encounter a formidable array of cellular stressors (hypoxia, limiting nutrients, proteotoxic and oxidative stress) that are intrinsic to their anabolic metabolism in an expanding tumor (Ackerman & Simon, 2014). As tumor cells detach from the extracellular matrix, invade the surrounding stroma, enter the bloodstream and colonize distant organs, they confront additional stressors that threaten their survival (Mehlen & Puisieux, 2006). To overcome these cellular stressors, transformed cells exploit components of the conserved mammalian stress response, relying on deregulated expression of multifunctional molecular chaperone networks to promote cell viability in the “stressed” tumor microenvironment (Jolly & Morimoto, 2000). One such network is comprised of the small heat shock protein

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Conflict of interest

The authors declare that there are no conflicts of interest.

(sHsp) family (HspB1–HspB10 in humans) defined structurally by the presence of a conserved α -crystallin domain (Haslbeck & Vierling, 2015; Treweek et al., 2015). These ATP-independent molecular chaperones function as ancient sentinels of proteotoxic stress that preserve proteostasis by stabilizing non-native or misfolded proteins to prevent protein aggregation. In addition, they directly interact with components of the conserved apoptotic cell death machinery to prevent apoptosis induction (Bakthisaran et al., 2015). α B-crystallin/HspB5 is a widely expressed sHsp first identified in the lens that has been implicated in the pathogenesis of diverse diseases, including myopathies, neurodegenerative disorders, cataracts and cancer (Boelens, 2014). Although there have been several recent reviews highlighting new structural insights and its role in other diseases (Hochberg & Benesch, 2014; Thanos et al., 2014; van der Smagt et al., 2014; Bakthisaran et al., 2015; Haslbeck & Vierling, 2015; Haslbeck et al., 2015; Nagaraj et al., 2015; Treweek et al., 2015), the present review will focus on the mechanisms by which α B-crystallin inhibits cancer cell death, its emerging role as a pathogenic driver in metastasis, and therapeutic efforts to target α B-crystallin in cancer. Insights regarding the role of other sHsps, including Hsp27, in cancer have been reviewed elsewhere (Arrigo et al., 2007; Acunzo et al., 2014).

2. Ancient stress sentinels that promote cell survival

2.1. α B-crystallin gene (*CRYAB*)

The α B-crystallin gene (*CRYAB*) is located on human chromosome 11q22.3–q23.1 adjacent to the structurally related HspB2/MKBP gene (Ngo et al., 1989; Jeanpierre et al., 1993). These two sHsp genes are aligned head-to-head, sharing an 1111 base pair intergenic promoter that enables differential, orientation-specific transcriptional regulation of their mRNAs (Swamynathan & Piatigorsky, 2002; Doerwald et al., 2004). Notably, the α B-crystallin gene, but not the HspB2 gene, is robustly induced by cellular stress such as heat shock by Hsf1- and Hsf2-mediated transcriptional activation of conserved heat shock response elements in the intergenic promoter (Sugiyama et al., 2000; Sadamitsu et al., 2001; Aki et al., 2003; Shinkawa et al., 2011). In addition to its stress-inducible expression, α B-crystallin is highly expressed in the lens, retina, cardiac and skeletal muscle, brain, placenta, kidney, lungs, colon and other tissues (Sugiyama et al., 2000; Treweek et al., 2015).

2.2. Protein structure and oligomer assembly

The α B-crystallin protein is composed of 175 amino acids and is organized into the canonical sHsp domains: an N-terminal region of approximately 65 amino acid residues that includes the three major phosphorylations sites (Ser¹⁹, Ser⁴⁵ and Ser⁵⁹) and is poorly conserved across the family, a conserved α -crystallin domain of 84 amino acids, and a C-terminal region composed of 26 amino acids that is largely divergent among sHsps except for an Ile¹⁵⁹–X–Ile¹⁶¹ motif (Haslbeck & Vierling, 2015; Haslbeck et al., 2015; Treweek et al., 2015). Under physiologic conditions, α B-crystallin assembles into large, heterogeneous and highly dynamic oligomers composed of 12–32 monomers (420–980 kDa) that have made structural analyses a formidable challenge, although great progress has been made recently utilizing multiple state-of-the-art approaches. The molecular portrait that is emerging reveals that the α -crystallin domain plays a critical role in dimerization: monomers form an anti-parallel-sheet that resembles an immunoglobulin-like fold with β -

strands $\beta 6$ and $\beta 7$ on their respective monomers at the interface (Fig. 1A) (Bagneris et al., 2009; Jehle et al., 2010; Laganowsky et al., 2010; Haslbeck & Vierling, 2015; Treweek et al., 2015). Although the α -crystallin domain is necessary and sufficient for dimer formation, the C-terminal and N-terminal regions regulate oligomer assembly and disassembly (Haslbeck & Vierling, 2015; Haslbeck et al., 2015; Treweek et al., 2015). Specifically, the C-terminal Ile¹⁵⁹–X-Ile¹⁶¹ motif from one dimer inserts into a groove in an adjacent dimer's α -crystallin domain formed by the $\beta 4$ and $\beta 8$ strands (Fig. 1B), thereby facilitating oligomerization into tetramers or hexamers, while the N-terminal region contributes to the formation of larger oligomers (Jehle et al., 2010, 2011; Delbecq & Klevit, 2013; Haslbeck et al., 2015). In addition, stress-induced phosphorylation of the N-terminal Ser residues (Ser¹⁹, Ser⁴⁵ and Ser⁵⁹) and/or phosphomimetic mutants inhibit the formation of large oligomeric complexes, resulting in smaller oligomeric subunits with enhanced chaperone activity as described in the following paragraph (Ito et al., 2001; Koteiche & McHaourab, 2003; Ecroyd et al., 2007; Peschek et al., 2013). α B-crystallin also forms hetero-oligomers with other sHsps, including α A-crystallin, Hsp27 and Hsp20 (Sugiyama et al., 2000; Mymrikov et al., 2012; Aquilina et al., 2013).

2.3. Preserving proteostasis

Although the precise mechanisms by which α B-crystallin binds to non-native proteins, including the relevant protein domains, are poorly understood, the dynamic nature of α B-crystallin subunit assembly and disassembly is likely to play a critical role in its chaperone function (Haslbeck & Vierling, 2015; Haslbeck et al., 2015; Treweek et al., 2015). An emerging model is that the large oligomeric complexes represent a storage depot that undergoes stress-inducible disassembly to dimers and smaller oligomers that facilitate interaction with misfolded client proteins by exposing substrate binding domains that are inaccessible in the large oligomers, including N-terminal and C-terminal regions as well as the hydrophobic groove in the α -crystallin domain formed by the $\beta 4$ and $\beta 8$ strands that is exposed by dissociation of the C-terminal Ile¹⁵⁹–X-Ile¹⁶¹ motif from the adjacent dimer during oligomer disassembly (Giese & Vierling, 2002; Benesch et al., 2008; McHaourab et al., 2009; Haslbeck & Vierling, 2015; Treweek et al., 2015). Consistent with this idea, the sequence ⁷³DRFSVNLDVKHFSPEELKVK⁹² in the α -crystallin domain of α B-crystallin functions as a mini-chaperone to prevent protein aggregation (Bhattacharyya et al., 2006). The substrate bound dimer then reassembles into large oligomers that sequester the misfolded protein from other misfolded proteins, thereby preventing potentially cytotoxic intracellular protein aggregation (Haslbeck & Vierling, 2015; Treweek et al., 2015). Alternatively, the misfolded protein can be delivered to a refolding competent, ATP-dependent molecular chaperone such as Hsp70 to restore the native protein conformation (Ehrnsperger et al., 1997; Haslbeck & Vierling, 2015; Treweek et al., 2015). As such, α B-crystallin and related sHsps function as ancient, on-demand stress-inducible molecular chaperones that protect proteostasis. However, the seemingly promiscuous nature of these chaperone/substrate interactions, ATP-independence and limited structural data have led to the impression that α B-crystallin and other sHsps are less promising drug targets than other molecular chaperones such as Hsp90.

2.4. Subverting apoptotic cell death

In addition to its well-established role in preserving proteostasis, α B-crystallin directly interacts with conserved components of the apoptotic cell death machinery to inhibit cell death. On a cellular level, apoptosis is executed by two principal pathways: the intrinsic (mitochondrial) and the extrinsic (death receptor) pathways that converge on the proteolytic activation of procaspase-3, the executioner caspase that cleaves key proteins to initiate cell death (Cryns & Yuan, 1998; Logue & Martin, 2008). Intriguingly, α B-crystallin inhibits activation of this key cell death protease that lies at the convergence of both apoptotic pathways, thereby conferring protection against a broad range of apoptotic stimuli, including chemotherapy and other cytotoxic drugs, tumor necrosis factor (TNF)- α , TNF-related apoptosis-inducing ligand (TRAIL), growth factor deprivation, matrix detachment-induced apoptosis, hypoxia, hypertonic and oxidative stress, ultraviolet radiation and others (Kegel et al., 1996; Mehlen et al., 1996; Andley et al., 2000; Kamradt et al., 2001, 2002, 2005; Stegh et al., 2008; Ruan et al., 2011; Dou et al., 2012; Petrovic et al., 2013; van de Schootbrugge et al., 2014; Malin et al., 2015). Conversely, silencing α B-crystallin confers sensitivity to cell death stimuli (Kamradt et al., 2005; Moyano et al., 2006; Lee et al., 2012; Petrovic et al., 2013; Pereira et al., 2014; Malin et al., 2015). Procaspase-3 is activated by a two-step mechanism in which initiator caspases (caspase-9 in the intrinsic pathway or caspases-8 or -10 in the extrinsic pathway) cleave procaspase-3 between its large and small subunits to generate a p24 intermediate, which subsequently undergoes autoproteolytic cleavage to remove its N-terminal domain (Cryns & Yuan, 1998; Logue & Martin, 2008). Active caspase-3 is a tetramer composed of two large/small subunit heterodimers.

2.5. Inhibiting caspase-3 activation

α B-crystallin binds to procaspase-3 and the p24 intermediate to prevent its subsequent proteolytic processing and activation (Fig. 2) (Kamradt et al., 2001; Mao et al., 2001; Stegh et al., 2008). The interaction between α B-crystallin and procaspase-3/p24 is quite specific: α B-crystallin does not interact with procaspases-7 or -9 (Stegh et al., 2008). Notably, a myopathy-causing R120G mutant α B-crystallin that impairs its chaperone function or deletion of the C-terminal 14 amino acids attenuates its antiapoptotic function (Kamradt et al., 2005). These findings underscore that α B-crystallin inhibits caspase-3 activation by disrupting its proteolytic processing. However, the relevant interacting domains in α B-crystallin and caspase-3 have not been delineated.

2.6. Interaction with other apoptosis regulators

α B-crystallin also negatively regulates apical events in the intrinsic apoptotic pathway upstream of caspase-3 activation (Fig. 2). The intrinsic apoptotic pathway is initiated by genotoxic stress-dependent activation of the proapoptotic Bcl-2 family member Bax, which translocates to the mitochondria and induces mitochondrial outer membrane permeabilization, cytochrome c release, and Apaf-1-dependent activation of the initiator caspase-9 in the apoptosome (Cryns & Yuan, 1998; Logue & Martin, 2008). α B-crystallin binds to proapoptotic Bcl-2 family members Bax and Bcl-x_s to prevent their mitochondrial translocation, mitochondrial outer membrane permeabilization, cytochrome c release and subsequent caspase-3 activation (Mao et al., 2004; Hamann et al., 2013). The myopathy-

causing R120G mutant α B-crystallin binds less robustly to Bax and Bcl-x_s and exerts diminished antiapoptotic activity (Mao et al., 2004). α B-crystallin also binds the p53 tumor suppressor gene product preventing its translocation to the mitochondria (Liu et al., 2007), which would likely inhibit its ability to induce Bax-dependent mitochondrial outer membrane permeabilization (Chipuk et al., 2004). In addition, α B-crystallin has been reported to promote p53 degradation by forming an Fbx4- α B-crystallin E3 ubiquitin ligase that marks p53 for proteasomal degradation (Jin et al., 2009). Taken together, these studies demonstrate that α B-crystallin directly binds to proapoptotic Bax or p53 (an activator of Bax) to inhibit mitochondrial outer membrane permeabilization and subsequent caspase activation.

2.7. Suppressing oxidative and endoplasmic reticulum stress

α B-crystallin is induced by oxidative stress and inhibits apoptosis induced by reactive oxygen species (ROS) by increasing intracellular glutathione concentrations, which directly buffer ROS levels and neutralize ROS-dependent protein oxidation and lipid peroxidation (Mehlen et al., 1996; Shin et al., 2008; Fittipaldi et al., 2015). Similarly, endoplasmic reticulum (ER) stress due to the accumulation of misfolded proteins in the ER lumen activates the unfolded protein response (UPR), which leads to α B-crystallin induction by the IRE1 and ATF6 arms of the UPR (Ruan et al., 2011). α B-crystallin inhibits ER stress-induced apoptosis by attenuating Bax induction and mitochondrial membrane permeability transition (Dou et al., 2012). Notably ER stress also leads to diminished glutathione levels and resultant oxidative stress, which may represent another molecular target for the cytoprotective effects of α B-crystallin in response to ER stress. These findings suggest that α B-crystallin is induced by multiple cellular stressors and acts to restore homeostasis by limiting the cytotoxicity of these stressors.

2.8. Effect of posttranslational modifications on the antiapoptotic function of α B-crystallin

Given that α B-crystallin undergoes a number of posttranslational modifications in response to stress (*e.g.*, site-specific phosphorylation, acetylation and glycosylation) (Haslbeck & Vierling, 2015; Haslbeck et al., 2015; Treweek et al., 2015), it is imperative to examine how these posttranslational modifications impact its antiapoptotic function. A pseudophosphorylation α B-crystallin mutant in which each of the Serine residues in the N-terminal domain that undergoes stress-induced phosphophorylation is replaced with a glutamic acid residue (S19E/S45E/S59E) is impaired in its ability to inhibit caspase-3 activation and apoptosis (Kamradt et al., 2005). Consistent with these latter findings, WT α B-crystallin, but not an S59E α B-crystallin mutant, protects breast cancer cells against vinblastine-induced apoptosis (Launay et al., 2010). Indeed, the S59E α B-crystallin mutant selectively binds Bcl-2 to prevent its translocation to the mitochondria, thereby facilitating mitochondrial outer membrane permeabilization, the first step in the intrinsic apoptotic pathway. However, one report in cardiac myocytes demonstrated enhanced caspase-3 inhibition and cytoprotection against hypoxia by the S59E α B-crystallin mutant (Morrison et al., 2003). Additional posttranslational modifications of α B-crystallin include acetylation of Lys⁹² and O-linked attachment of the monosaccharide β -N-acetylglucosamine to Thr¹⁷⁰ that have been linked to enhanced antiapoptotic activity (Krishnamoorthy et al., 2013; Nahomi et al., 2013). Clearly, our understanding of how these posttranslational

modifications affect its antiapoptotic function is incomplete and will require additional studies to determine whether these modifications have cell-type or stimulus-specific effects.

3. Deregulated expression in cancer

3.1. An emerging biomarker of poor clinical outcomes

α B-crystallin is abundantly expressed in a multitude of solid tumors, including malignant glioblastomas, osteosarcoma, retinoblastoma and carcinomas of the breast, prostate, ovary, colon, liver, lung (non-small cell), head and neck, and thyroid (Aoyama et al., 1993; Wulfschle et al., 2002; Stronach et al., 2003; Chelouche-Lev et al., 2004; Shi et al., 2004, 2014; Chin et al., 2005; Moyano et al., 2006; Goplen et al., 2010; Kim et al., 2011; Mao et al., 2012; Huang et al., 2013; Volkmann et al., 2013; Davidov et al., 2014; Qin et al., 2014; Yilmaz et al., 2015). In many cases, α B-crystallin levels are higher in the tumor tissue than the surrounding non-tumor tissue (Wulfschle et al., 2002; Shi et al., 2004, 2014; Mao et al., 2012; Qin et al., 2014; Yilmaz et al., 2015). Typically, α B-crystallin expression is associated with aggressive tumor characteristics and poor clinical outcomes. For example, in non-small cell lung cancer, α B-crystallin protein expression correlates with tumor-node-metastasis (TNM) stage and is an independent prognostic factor for poor overall survival (Qin et al., 2014). A second study reported that nuclear expression of α B-crystallin protein in non-small cell lung cancer is an independent biomarker for inferior survival (Cherneva et al., 2010). In colorectal cancer, α B-crystallin protein expression is an independent predictor of distant metastasis and inferior survival (Shi et al., 2014). Additionally, α B-crystallin protein expression correlates with shorter time to relapse or distant metastasis and poor survival in head and neck squamous cell carcinomas (Chin et al., 2005; van de Schootbrugge et al., 2013a). Similar associations between primary tumor expression of α B-crystallin and poor clinical outcomes have been reported in hepatocellular carcinoma, ovarian cancer, laryngeal carcinoma and other solid tumors (Mao et al., 2012; Huang et al., 2013; Volkmann et al., 2013). Although these correlative studies by their very nature do not prove causality, the preponderance of data linking α B-crystallin to tumor progression, metastasis and poor survival strongly suggests that α B-crystallin may contribute functionally to these outcomes.

Perhaps the strongest clinical data linking α B-crystallin to malignancy and tumor progression comes from studies in breast cancer. α B-crystallin is expressed at greater than 14-fold higher levels in preinvasive ductal carcinoma in situ (DCIS) compared with matched normal breast tissue (Wulfschle et al., 2002). In normal adult breast tissue, α B-crystallin is expressed in a subset of myoepithelial cells in the basal or outer epithelial layer, but not in luminal epithelial cells that secrete milk into the lumen (Moyano et al., 2006; Sitterding et al., 2008). Intriguingly, the α B-crystallin gene (*CRYAB*) is commonly expressed in a clinically aggressive subtype of human breast carcinomas that express genes characteristic of basal epithelial genes (Perou et al., 2000; Moyano et al., 2006). These basal-like breast cancers typically lack expression of the estrogen receptor, progesterone receptor and HER-2 and are sometimes referred to as triple-negative breast carcinomas (TNBC) (Yehiely et al., 2006; Toft & Cryns, 2011). Multiple studies have confirmed the association of α B-crystallin protein expression with expression of basal markers (e.g., cytokeratin 5 and 14) and TNBC, which is associated with poor survival due to early relapse in the lungs and brain (Moyano et

al., 2006; Ivanov et al., 2008; Smid et al., 2008; Kennecke et al., 2010; Kim et al., 2011; Toft & Cryns, 2011; Tsang et al., 2012; Malin et al., 2014). α B-crystallin is also commonly expressed in metaplastic carcinomas, a rare histologic subtype with spindle or sarcomatoid features that express basal markers and are likely to represent a distinctive subset of basal-like breast tumors (Reis-Filho et al., 2006; Sitterding et al., 2008; Chan et al., 2011). α B-crystallin expression in primary breast carcinomas is associated with poor disease-free and overall survival, lymph node metastases, and inferior response to presurgery (neoadjuvant) chemotherapy (Chelouche-Lev et al., 2004; Moyano et al., 2006; Ivanov et al., 2008; Kim et al., 2011; Malin et al., 2014). α B-crystallin is expressed in brain metastases from breast cancer patients, and its expression in primary breast carcinomas in a cohort of women with breast cancer who developed brain metastases was associated with poor overall survival and survival after brain metastasis (Malin et al., 2014). More recently, *CRYAB* gene expression in breast cancer has been shown to be an independent predictor of brain metastasis as the first site of distant relapse, while α B-crystallin protein expression is an independent predictor of brain metastasis (first site or any occurrence) and shorter survival after diagnosis of brain metastasis (Voduc et al., 2015). On a molecular level, α B-crystallin protein expression in primary breast carcinomas correlates with expression of Ki67 (a marker of proliferation), EGFR, phospho-ERK, phospho-mTOR and phospho-AKT (van de Schootbrugge et al., 2013b; Koletsa et al., 2014). Collectively, the robust association between α B-crystallin expression and TNBC as well as multiple poor clinical outcomes (inferior survival, chemotherapy resistance and brain metastases) suggests that α B-crystallin may contribute to tumor progression.

Although the overwhelming majority of clinical data have linked α B-crystallin to an aggressive tumor phenotype, there are a few notable exceptions that have been reported. *CRYAB* mRNA and α B-crystallin protein levels were reported to be reduced in a small study of highly aggressive anaplastic thyroid carcinomas relative to benign goiters (Mineva et al., 2005). Similarly, low *CRYAB* gene expression was associated with inferior survival in a small cohort of 18 ovarian carcinoma cases (Stronach et al., 2003), although in larger study of 103 ovarian cancer patients, α B-crystallin protein expression was associated with poor relapse-free and overall survival (Volkman et al., 2013). In a few other studies of diverse tumor types, α B-crystallin expression was not significantly associated with prognosis (Boslooper et al., 2008; Kabbage et al., 2012; Yilmaz et al., 2015). It remains to be determined whether these divergent results reflect differences in study design and/or tumor type.

3.2. Regulation of α B-crystallin expression by cellular stress in the tumor microenvironment

Despite the multitude of studies demonstrating deregulated or enhanced expression of α B-crystallin in solid tumors, there is no reported evidence that the α B-crystallin gene (*CRYAB*) is amplified or mutated in cancer and functions as an oncogene. On the contrary, α B-crystallin expression is induced by multiple stressors that are inherent to the malignant state and/or the microenvironment confronted by the expanding tumor. In response to genotoxic stress, the p53 tumor suppressor gene product transcriptionally regulates *CRYAB* expression, either directly by binding to one or two conserved p53 response elements in its

intergenic promoter or indirectly by inducing the expression of the p53 family member

Np73, which in turn transcriptionally regulates *CRYAB* expression (Watanabe et al., 2009; Evans et al., 2010; Liu et al., 2014). These studies link α B-crystallin gene expression to genotoxic stress via the action of p53. In addition, the Ets1 oncogenic transcription factor, which is highly expressed in TNBC and correlates with poor survival, directly binds to an ETS response element in the *CRYAB* promoter to activate transcription, thereby providing an additional mechanism for the robust expression of α B-crystallin in TNBC (Bosman et al., 2010). In malignant glioblastomas, the Bcl2L12 oncogene on chromosome 19q13 is highly expressed and induces robust expression of α B-crystallin protein by mechanisms that have not been elucidated (Stegh et al., 2007, 2008). Moreover, an oncogenic mutation in the tricarboxylic acid cycle enzyme isocitrate dehydrogenase-1 (R132H) that is commonly observed in gliomas drives enhanced α B-crystallin gene and protein expression (Avliyakov et al., 2014). In contrast, inactivating mutations of the tuberous sclerosis tumor suppressor genes (*Tsc1* or *Tsc2*), increase α B-crystallin gene and protein expression by an mTORC2- and NF κ B-dependent mechanism (Wang et al., 2014). Collectively, these findings indicate that oncogenic stress results in deregulated expression of α B-crystallin, which would be expected to mitigate this stress (see the next section).

α B-crystallin is abundantly expressed in hypoxic regions of the expanding tumor, although in cell-based assays reoxygenation and subsequent generation of ROS, rather than hypoxia, activate α B-crystallin gene and protein expression (van de Schootbrugge et al., 2014). Metastatic carcinoma cells detach from the extracellular matrix in order to invade the surrounding stroma and enter the circulation *en route* to establishing metastases in distant organs (Nguyen et al., 2009). Matrix detachment disrupts integrin-mediated survival signals and typically activates matrix detachment-induced apoptosis or “anoikis” in non-transformed epithelial cells (Frisch & Screaton, 2001). Intriguingly, matrix detachment robustly induces α B-crystallin mRNA and protein levels by inhibiting ERK activity (Malin et al., 2015). Indeed, ERK negatively regulates α B-crystallin gene and protein expression by mechanisms that have yet to be determined. Finally, the anabolic metabolism of rapidly growing tumors and requisite demands for ongoing protein synthesis lead to ER stress, which as already noted results in increased α B-crystallin expression via the IRE1 and ATF6 arms of the UPR (Ruan et al., 2011). Taken together, these studies illustrate that multiple cellular stressors intrinsic to the tumor microenvironment enhance α B-crystallin expression and suggest that targeting this deregulated expression may represent a unique therapeutic strategy.

4. Metastasis enabler

4.1. α B-crystallin promotes oncogenic transformation

Consistent with its enhanced expression in solid tumors, several studies have demonstrated an important functional role for α B-crystallin in oncogenic transformation and/or tumor progression. Overexpression of wild-type α B-crystallin in immortalized but non-transformed breast epithelial cells promoted oncogenic transformation as determined by luminal filling of mammary acinar-like structures in three-dimensional tissue culture models, enhanced growth factor- and anchorage-independent growth, and ability to initiate mammary tumors *in vivo* (Moyano et al., 2006). In contrast, a pseudophosphorylation (S19E/S45E/

S59E) α B-crystallin mutant that is impaired in its ability to inhibit caspase-3 activation and apoptosis was unable to transform breast epithelial cells, thereby indicating that oncogenic transformation by α B-crystallin is dependent on its antiapoptotic activity. Similarly, overexpression of wild-type α B-crystallin, but not the S19E/S45E/S59E mutant, transformed human glioma cells as determined by glioma formation in an orthotopic murine model (Stegh et al., 2008). α B-crystallin also promotes oncogenic transformation in the setting of combined p53 and Rb inactivation (Petrovic et al., 2013). Rb inactivation primes cancer cells for apoptosis by augmenting expression of multiple procaspases, and α B-crystallin specifically antagonizes this effect by inhibiting procaspase-3 activation (Nahle et al., 2002; Petrovic et al., 2013). Moreover, silencing α B-crystallin in human TNBC cells, which express mutant p53 and lack expression of Rb, increases their sensitivity to chemotherapy-induced apoptosis (Petrovic et al., 2013), suggesting that inhibiting α B-crystallin might be an effective strategy to enhance the therapeutic efficacy of chemotherapy.

4.2. α B-crystallin promotes metastasis in vivo

More recently, a functional role for α B-crystallin in metastasis has been established in murine models. Specifically, α B-crystallin promotes lung and brain metastasis in orthotopic models of TNBC in which fluorescently labeled human TNBC metastasize from the mammary gland to distant organs including the lungs and brain (Malin et al., 2014, 2015). Intriguingly, modulating α B-crystallin levels in established TNBC cells did not affect primary tumor growth in these studies, suggesting that α B-crystallin specifically regulates one or more steps in the metastatic cascade as tumor cells detach from the extracellular matrix, invade the surrounding stroma, intravasate into the circulation, survive as circulating tumors cells, extravasate into distant organs and colonize the organ (Nguyen et al., 2009; Malin et al., 2014, 2015). Since metastatic tumor cells must overcome apoptosis at each of these steps, the antiapoptotic function of α B-crystallin is likely tightly linked to its prometastatic actions (Mehlen & Puisieux, 2006; Malin et al., 2014, 2015). Moreover, α B-crystallin promotes epithelial-to-mesenchymal transition (EMT), a fundamental process linked to metastasis, and enhances lung metastasis in a murine model of human hepatocellular carcinoma (Huang et al., 2013; Tam & Weinberg, 2013). Notably, the observation that silencing α B-crystallin in multiple metastatic murine models inhibits lung and/or brain metastasis strongly supports the rationale for targeting α B-crystallin as a novel antimetastatic strategy (Huang et al., 2013; Malin et al., 2014, 2015). In the remainder of this section, we will discuss the evidence linking α B-crystallin to each of the steps in the metastatic cascade.

4.3. Epithelial-to-mesenchymal transition

Carcinoma cells undergoing EMT acquire mesenchymal characteristics that promote cell migration and invasion and facilitate metastasis to distant organs (Tam & Weinberg, 2013). The TGF- β -Smad signaling pathway regulates the expression of several transcription factors such as Snail, Slug and Twist that drive the EMT phenotype. Overexpression of α B-crystallin in epithelial cells and fibroblasts increases the nuclear localization of Smad4 by antagonizing its mono-ubiquitination and subsequent nuclear export, thereby promoting activation of TGF- β downstream target genes (Bellaye et al., 2014). Additionally, overexpression of α B-crystallin in human hepatocellular carcinoma cells induces EMT by

activating ERK by a 14-3-3 ζ -dependent mechanism that enhances Slug expression (Huang et al., 2013). These studies suggest that α B-crystallin regulates core components of the EMT program to facilitate tumor progression.

4.4. Cell migration and invasion

Several studies have demonstrated that α B-crystallin promotes cell migration and invasion. α B-crystallin expression is more prominent in the infiltrative edge of malignant gliomas and the leading edge of migrating lens epithelial cells (Maddala & Rao, 2005; Goplen et al., 2010). Overexpression of wild-type α B-crystallin, but not the S19E/S45E/S59E mutant, in breast epithelial cells enhances cell migration and invasion by a MEK-dependent mechanism (Moyano et al., 2006). Consistent with these findings, α B-crystallin overexpression in renal cell carcinoma cells increases cell migration, while silencing α B-crystallin in head and neck squamous cell carcinoma cells inhibits cell migration (Ho et al., 2013; van de Schootbrugge et al., 2013a). In addition, α B-crystallin regulates invasion in human hepatocellular carcinoma cells (Huang et al., 2013). Although the mechanisms underlying these effects on cell migration/invasion have not been clearly defined, the ability of α B-crystallin to interact with and stabilize the focal adhesion kinase (FAK), intermediate filaments and microtubules may contribute to its promigratory actions (Djabali et al., 1997, 1999; Ghosh et al., 2007a; Pereira et al., 2014).

4.5. Anoikis resistance

Normal epithelial cells require integrin-mediated survival signals initiated by attachment to the extracellular matrix and undergo caspase-dependent anoikis upon matrix detachment (Frisch & Screaton, 2001). As such, the acquisition of anoikis resistance is a hallmark of metastatic carcinoma cells that enables them to survive matrix detachment as they disseminate as circulating tumor cells (Guadamillas et al., 2011). Matrix detachment induces α B-crystallin gene and protein expression by an ERK-dependent mechanism (Malin et al., 2015). Specifically, suppression of ERK activity is both necessary and sufficient for matrix detachment-initiated α B-crystallin induction. Consistent with its antiapoptotic function, silencing α B-crystallin in metastatic carcinoma cells sensitizes them to matrix detachment-induced caspase activation and anoikis but does not affect their viability in adherent culture. Notably, silencing α B-crystallin in these metastatic carcinoma cells reduced the viability of circulating tumor cells and suppressed lung metastases in orthotopic murine models of TNBC but had minimal or no impact on mammary tumor growth (Malin et al., 2015). Consistent with these findings, α B-crystallin is highly expressed in circulating tumor cells in canines with mammary tumors (da Costa et al., 2012). These results point to α B-crystallin as a key regulator of anoikis resistance and a promising target to activate anoikis and suppress metastasis by inhibiting its expression or antagonizing its function.

4.6. Angiogenesis

Tumor angiogenesis, the process by which tumors form new blood vessels from established ones, plays a critical role in tumor growth and metastasis (Kerbel, 2008; Bielenberg & Zetter, 2015). Many tumors produce proangiogenic factors such as VEGF-A (abbreviated VEGF), which promotes tumor progression by (1) paracrine actions on endothelial cells, resulting in increased proliferation and migration as well as enhanced vascular permeability;

and (2) autocrine actions on tumor cells, which express VEGF receptors. α B-crystallin is induced during tubular morphogenesis of endothelial cells and promotes endothelial cell survival during this process (Dimberg et al., 2008). Consistent with these *in vitro* findings, transplanted teratocarcinomas in α B-crystallin-deficient mice exhibited defective vascularization with leaky blood vessels and enhanced tumor cell death compared to tumors in wild-type α B-crystallin mice, although tumor size was not affected. Moreover, α B-crystallin binds to VEGF, protects it from proteolytic degradation and increases VEGF secretion in retinal pigment epithelial cells, cancer cells and endothelial cells co-cultured with cancer cells (Kase et al., 2010; Ruan et al., 2011; van de Schootbrugge et al., 2013a). These studies suggest that α B-crystallin may modulate tumor angiogenesis by its combined effects on endothelial cells (promotion of cell survival) and tumor cells (enhanced VEGF expression/secretion with paracrine effects on endothelial cells and autocrine effects on tumor cells). It remains to be determined whether α B-crystallin expression in tumor cells affects their sensitivity to antiangiogenic therapies targeting VEGF (e.g., the humanized VEGF monoclonal antibody bevacizumab) and receptor tyrosine kinase inhibitors (e.g., sunitinib and sorafenib).

4.7. Intravasation

Metastatic tumor cells invade the surrounding stroma and enter the circulation by the process of intravasation (Nguyen et al., 2009). Although there is no reported *in vivo* data regarding the impact of α B-crystallin on intravasation, α B-crystallin promotes transendothelial migration of cultured TNBC cells (Malin et al., 2014), suggesting a potential functional role for α B-crystallin in intravasation.

4.8. Extravasation

Circulating tumor cells extravasate from the circulation by adhering to endothelial cells or the adjacent basement membrane in organ capillaries and moving through gaps in the capillary wall (Nguyen et al., 2009). Consistent with a potential role in extravasation, α B-crystallin promotes adhesion to human brain microvascular endothelial cells (HBMECs) by an α 3 β 1 integrin-dependent mechanism and enhances penetration through a primary HBMEC/human astrocyte co-culture model of the blood–brain barrier (Malin et al., 2014). It is unclear whether these effects are restricted to the brain microenvironment and whether these effects are observed *in vivo*.

4.9. Organ-specific colonization

In murine models, α B-crystallin promotes metastasis from the mammary gland to multiple organs including the lungs, liver, bone and brain (Malin et al., 2014, 2015). However, clinical studies indicate a strong association between α B-crystallin expression in primary breast carcinomas and the development of brain metastasis (Voduc et al., 2015). Furthermore, α B-crystallin promotes lung metastasis in murine models of hepatocellular carcinoma (Huang et al., 2013). Additional studies are needed to determine whether α B-crystallin expression in primary tumors promotes sites-specific metastasis to the brain, lungs and/or other sites and the underlying mechanism(s) of its potential metastatic tissue tropism.

Overall, α B-crystallin is induced by a wide variety of stressors confronting transformed cells as they disseminate from the primary tumor and promotes many of the steps in the metastatic cascade. Viewed from this context, α B-crystallin enables transformed cells to adapt to cellular stress during tumor progression (matrix detachment, cell migration/invasion, intravasation, survival in circulation, extravasation, and organ colonization). As such, it functions as a “metastasis enabler” that distinguishes it from genetic alterations in oncogenes or tumor suppressor genes that drive metastasis (Fig. 3).

5. Cancer drug target

5.1. Rationale for targeting α B-crystallin in cancer

The emerging portrait of α B-crystallin as a metastasis enabler that counteracts oncogenic stress as transformed cells disseminate to distant organs strongly suggests that targeting α B-crystallin may represent a promising strategy to selectively suppress metastasis. Conventional chemotherapy is ineffective against metastatic solid tumors, which typically follow a progressive and ultimately fatal course, making it imperative to develop novel therapeutic approaches (Coley, 2008). Several observations support α B-crystallin as a propitious molecular target for antimetastatic therapies. First, α B-crystallin is highly expressed in poor-prognosis solid tumors (Chin et al., 2005; Moyano et al., 2006; Cherneva et al., 2010; Kim et al., 2011; Mao et al., 2012; Huang et al., 2013; van de Schootbrugge et al., 2013a; Volkmann et al., 2013; Malin et al., 2014; Qin et al., 2014; Shi et al., 2014). Second, α B-crystallin confers apoptosis-resistance to transformed cells, and silencing α B-crystallin enhances their sensitivity to apoptosis induction by many stimuli (Mehlen et al., 1996; Kamradt et al., 2002, 2005; Stegh et al., 2008; Ruan et al., 2011; Petrovic et al., 2013; van de Schootbrugge et al., 2014; Malin et al., 2015). Third, silencing α B-crystallin in TNBC cells inhibits attachment to endothelial cells, transendothelial migration, penetration through an *in vitro* blood–brain barrier model, and circulating tumor cell survival (Malin et al., 2014, 2015). Fourth, silencing α B-crystallin in diverse human cancer cells inhibits metastasis to the lungs and/or brain in murine models (Huang et al., 2013; Malin et al., 2014, 2015). Fifth, because α B-crystallin plays a key role in maintaining cell survival in both cancer cells and endothelial cells, inhibition of α B-crystallin would likely have direct antitumor effects and antiangiogenic actions, respectively (Mao et al., 2004; Moyano et al., 2006; Dimberg et al., 2008; Stegh et al., 2008; Kase et al., 2010; Launay et al., 2010; Ruan et al., 2011; Petrovic et al., 2013). Hence, strategies aimed at reducing α B-crystallin expression or inhibiting its actions would be predicted to suppress tumor progression and metastasis.

5.2. Inhibiting α B-crystallin expression

Although several drugs that activate cellular stress and induce α B-crystallin expression have been described, no selective inhibitors of α B-crystallin expression have been reported. Targeting the conserved stress response transcription factor Hsf1 represents a potential non-selective strategy to downregulate α B-crystallin expression in tumor cells, although additional Hsf1-regulated heat shock proteins would also be downregulated by this approach. Hsf1 promotes cell survival in the setting of oncogenic stress and is highly expressed in clinically aggressive solid tumors that are associated with poor survival (Dai et

al., 2007; Mendillo et al., 2012). Several small molecule inhibitors of Hsf1 have been identified and demonstrated to have antitumor effects in pre-clinical cell-based assays and murine models (Whitesell & Lindquist, 2009). While such global inhibition of the heat shock response may enhance its therapeutic efficacy in cancer, the sustained downregulation of multiple heat shock proteins would also be predicted to augment systemic toxicity.

5.3. Disrupting α B-crystallin–client interactions

To date, efforts to inhibit α B-crystallin for therapeutic purposes in cancer have focused on disrupting protein-protein interactions between this molecular chaperone and its client(s). Several peptide sequences in the N-terminal and α -crystallin domains of α B-crystallin corresponding to β -strands β 3, β 7, β 8 and β 9 have been identified that interact with VEGF, FGF-2, NGF- β , insulin and β -catenin (Ghosh et al., 2007b). These peptides also inhibit thermal aggregation of VEGF protein, suggesting that the client interacting and molecular chaperone protein domains may at least partly overlap. Subsequent studies employing α B-crystallin decoy peptides identified the β 7 and β 9 strands in α B-crystallin as the primary interacting domains with VEGF (Chen et al., 2014). More than 100,000 small molecules from the NCI Developmental Therapeutics Program were analyzed by molecular docking simulation to the pocket formed by the β 7 and β 9 strands in α B-crystallin. This simulation screen led to the identification of 40 compounds that were subsequently screened for cytotoxicity against TNBC cells, resulting in 4 lead compounds. One of these lead compounds, NCI-41356, a (2S,3R)-3-methylglutamic acid hydrochloride salt, disrupted the interaction between α B-crystallin and VEGF and inhibited the expression and secretion of VEGF in TNBC cells without altering α B-crystallin expression. Moreover, NCI-41356 inhibited cell growth, cell migration and the proangiogenic effects of TNBC cells on endothelial cells in the 10–50 μ M range. A single dose of NCI-41356 (0.2 g/kg by intraperitoneal injection) inhibited mammary tumor growth and tumor angiogenesis in a murine model of TNBC (Chen et al., 2014). These results provide proof-of-principle preclinical evidence for targeting α B-crystallin as a therapeutic strategy in cancer. Clearly, structure–function analyses and compound optimization would be needed to increase its therapeutic efficacy. Given that the β 7 and β 9 strands in α B-crystallin also mediate interaction with other clients and contribute to its chaperone activity (Ghosh et al., 2007b), the antitumor effects of NCI-41356 may also reflect these additional VEGF-independent actions. Moreover, the potential impact of this small molecule on tumor progression and metastasis was not reported. Nevertheless, disrupting the intracellular α B-crystallin–VEGF interaction, which subsequently reduces VEGF expression and secretion, provides a potentially new strategy to target tumor angiogenesis.

6. Conclusion

Deregulated expression of the molecular chaperone α B-crystallin is observed in a wide range of solid tumors and is associated with poor clinical outcomes in the vast majority of studies. Recent preclinical studies have demonstrated a pathogenic role for α B-crystallin in enabling tumor cells to survive tumor microenvironmental stressors and overcome many of the barriers tumor cells encounter as they disseminate from the primary tumor, invade the surrounding stroma, enter the bloodstream and colonize distant organs such as the lungs and

brain. Silencing α B-crystallin in tumor cells results in robust, multifaceted antitumor effects, including induction of apoptosis, inhibition of cell migration and invasion, reduction in circulating tumor cells and suppression of distant metastases. As such, α B-crystallin is a prime molecular target for antimetastatic therapies. Emerging strategies to target α B-crystallin by disrupting interactions with cancer-relevant clients such as VEGF provide proof-of-principle preclinical evidence supporting the feasibility of drug discovery efforts to rationally design inhibitors of this malignant chaperone.

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Abbreviations

CRYAB	α B-crystallin gene
EMT	epithelial-to-mesenchymal transition
ER	endoplasmic reticulum
sHsp	small heat shock protein
HBMECs	human brain microvascular endothelial cells
ROS	reactive oxygen species
TNBC	triple-negative breast cancer
UPR	unfolded protein response

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Fig. 1. Structure of α B-crystallin: an emerging portrait. (A) Ribbon diagram of the NMR structure of the α B-crystallin dimer. (B) Ribbon diagram demonstrating that oligomerization is facilitated by the C-terminal Ile¹⁵⁹-X-Ile¹⁶¹ motif from one dimer (blue) inserting into a groove in an adjacent dimer's α -crystallin domain formed by the β 4 and β 8 strands (red). Adapted by permission from Macmillan Publishers Ltd (Jehle et al., 2010).

Fig. 2.

αB-crystallin negatively regulates multiple steps in the intrinsic (mitochondrial) apoptotic pathway. In the intrinsic apoptotic pathway, genotoxic stress results in p53-dependent activation of the proapoptotic Bcl-2 family member Bax, which translocates to the mitochondria and induces mitochondrial outer membrane permeabilization, cytochrome c (abbreviated “c”) release, and Apaf-1-dependent activation of caspase-9 in the apoptosome. Caspase-9 in turn activates the executioner caspase-3. αB-crystallin binds to p53 to inhibit Bax activation and also binds to Bax to block its mitochondrial translocation. In addition, αB-crystallin binds to procaspase-3 and the p24 intermediate to suppress proteolytic activation of caspase-3.

Fig. 3. α B-crystallin as a multifaceted metastasis enabler. Potential mechanisms by which α B-crystallin promotes metastasis to distant organs include apoptosis-resistance, epithelial-to-mesenchymal transition (EMT), cell migration/invasion, resistance to matrix detachment-induced cell death (anoikis), angiogenesis, intravasation, extravasation and organ colonization.