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Hsp70 in cancer: back to the future

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Abstract

Mechanistic studies from cell culture and animal models have revealed critical roles for the heat shock protein Hsp70 in cancer initiation and progression. Surprisingly, many effects of Hsp70 on cancer have not been related to its chaperone activity, but rather to its role(s) in regulating cell signaling. A major factor that directs Hsp70 signaling activity appears to be the co-chaperone Bag3. Here, we review these recent breakthroughs, and how these discoveries drive drug development efforts.

Keywords

BAG-3; cancer transformation and progression; senescence; proteotoxicity

Introduction

In this review we provide an overview of the roles of Hsp70 in cancer, spanning the initial observations that the levels of the heat shock protein Hsp70 are elevated in many human tumors, to dissecting mechanisms of its action in cancer, and finally to development novel anticancer compounds.

Hsp70 is a founding member of the highly conserved Hsp70 family of molecular chaperones, which are found in every membranous organelle of all cells¹. There are two abundant family members found in the cytosol of mammalian cells, a constitutively expressed HSPA8 (Hsc70, Hsc73) and an inducible HSPA1A (Hsp70, Hsp72). Several minor family members also exist (e.g. GRP75 or GRP78), but will not be discussed here. Hsc73 is an indispensable chaperone, which under homeostatic conditions is involved in folding, translocation across membranes, and degradation of proteins². Unlike Hsc73, Hsp70 is normally kept at low levels, and is induced under protein damaging conditions such as heat shock, oxidative stress, hypoxia or heavy metals, functioning to provide resistance to a variety of proteotoxic stresses^{3,4}. Little is known about the functional specificity of Hsp70 family members, and until recently it has been assumed that these proteins function redundantly. Recent observations, however, suggest that these proteins differ in their ability to interact with the ubiquitin ligase CHIP, leading to differential effects on protein degradation, such as the degradation of tau, an important factor contributing to Alzheimer's disease⁵. With cancer there also appears to be specificity for Hsp70 family members, as depletion or knockout of Hsp70 leads to profound anti-cancer effects, despite

of the presence of Hsc73 (see below), though the molecular mechanisms underlying this specificity remain obscure.

Hsp70 is overexpressed in cancers, but not always

About two decades ago Daniel Ciocca and colleagues reported that expression of Hsp70 is highly elevated in breast cancer ⁶. Later this observation was extended for other types of tumors, including colon ⁷, liver ^{8, 9}, prostate ^{10, 11}, esophagus ¹², cervix ^{13, 14} (see also ¹⁵ for review). These findings led to numerous attempts to develop Hsp70 as a biomarker of tumor stage, metastases, or prognosis. In several human cancers, prognostic significance of Hsp70 has proven quite remarkable, and independent on others prognostic factors ^{16, 17, 18, 19, 20}. For example, in breast cancer without regional metastases at the time of diagnoses, 70% of patients with low levels of Hsp70 expression survive for 5 years, comparing with 30% survival of patients with high levels of Hsp70 expression ⁶. Despite substantial work in this field, Hsp70 has not yet been accepted as a clinical biomarker of any cancer parameter. Nevertheless, these studies have boosted investigations into the role(s) of Hsp70 in cancer, and have led to important developments in our understanding of etiology of malignancies, defining new avenues of drug design.

Though Hsp70 is overexpressed in most human cancers, there are some tumor types where lower expression of Hsp70 is observed compared to adjusted normal tissues, and correlation between Hsp70 levels and survival is lacking (e.g., in renal cancer ²¹, or cervix ¹⁴). Moreover, an inverse correlation between Hsp70 expression and patient's prognosis is observed in some cancers, such as squamous cell carcinoma ²², cholangiocarcinoma ²³, oral ²⁴, or lung cancer ²⁵.

One possible explanation for the negative correlation is that Hsp70 can suppress inflammation, likely by inhibiting the NF- κ B pathway ^{27–30}. Accordingly, in cancers where the inflammatory component is critical for their development, a *decrease* rather than *increase* in the levels of Hsp70 would promote cancer. Indeed, in mouse colon carcinoma caused by a carcinogen DSS, which is accompanied by severe inflammation, knockout of Hsp70 increased inflammatory cytokines and aggravated cancer ³¹.

Another possible role for Hsp70 in suppression of cancer is related to its immune-modulating properties ³². For instance, in squamous cell carcinoma, where high expression of Hsp70 associates with a good prognosis, there was a significant positive correlation between Hsp70 levels and lymphocyte infiltration which usually indicates higher anti-tumor immune response and is a favorable prognostic factor ²². The immune-modulating activity of Hsp70 is related to its *extracellular* function as immune stimulator (see ³² for review). Indeed, extracellular Hsp70 may activate innate immune system and can be used as adjuvant for tumor antigens, the property which is currently widely used in development of anticancer vaccines ^{33, 34}. Thus, one may suggest that in tumors with inverse correlation between expression Hsp70 and prognosis, stronger immune response to Hsp70-tumor antigen complexes (released from cells or presented on the cell surface) is involved. Discussion of immune-modulating functions of Hsp70, including the role of extracellular Hsp70 in innate immunity and membrane-associated Hsp70 in sensitivity to NK cells, is out of the scope of

this review, and here we will discuss cancers whose progression associates with elevated levels of Hsp70.

Dependence of tumor cells on Hsp70

Observations that certain types of tumors have elevated levels of Hsp70, which correlates with cancer grade and prognosis, suggested that Hsp70 could be involved in critical biochemical or genetic alterations that take place upon malignant transformation and further cancer development. In the mid-90s, when the phenomenon of apoptosis became the focus of biomedical research, it was discovered that Hsp70 potently suppresses apoptosis^{36–38}. Since tumor cells live under conditions of continuous stress, e.g. hypoxia, nutrient deprivation or low pH, all of which are potent inducers of apoptosis, development of tumor must require adaptations that suppress apoptosis³⁹. Accordingly, it was suggested that elevated levels of Hsp70 are critical to cancer cells to combat these harsh conditions and suppress apoptosis. These early studies demonstrated that, surprisingly, besides its molecular chaperone function, Hsp70 also plays a special role in apoptotic signal transduction. Indeed, Hsp70 could prevent apoptosis in response to a variety of conditions that do not cause protein damage, such as cisplatin^{40, 41} or TNF⁴². Moreover, mutants of Hsp70 lacking chaperone function still retained their ability to effectively suppress the TNF-induced apoptosis⁴² indicating that there must be a special function of Hsp70 in the apoptotic signal transduction. Indeed, it was found that Hsp70 suppresses apoptotic signaling at several points, including suppression of stress-activated kinases JNK and p38^{37, 38}, prevention of translocation of Bax or Bid to mitochondria^{42,43}, and suppression of the apoptosome formation⁴⁴. In line with these observations, depletion of Hsp70 sensitized to drug-induced apoptosis in myc-expressing lymphoid cells⁴⁵.

Apoptosis is a form of cell death which can be easily induced in lymphoid cells, but it plays a lesser role in epithelial cells from which most of human tumors originate. In some of these cancer cells of epithelial origin Hsp70 suppresses autophagic cell death which is independent on caspases and is not suppressed by Bcl-2⁴⁶. This form of cell death apparently associates with permeabilization of lysosomal membranes and release of lysosomal enzymes such as cathepsin⁴⁷. In spite of these conceptual advances, the lack of animal modeling makes it hard to conclude whether the anti-apoptotic or anti-autophagic death function of Hsp70 is important for cancer development.

While protection from harsh conditions of tumor microenvironment was initially considered the main reason for Hsp70 overexpression in tumors, a surprising observation was later made by the Jaattela group, that elevated levels of Hsp70 in unstressed cancer cells are required for their growth even under normal conditions^{46, 48}. This observation was further extended to several cancer cell lines, and now it is firmly established that tumor cells, in contrast to normal cells, require Hsp70 for their survival and growth (see ref^{49, 50} for recent review). In searching for the mechanism of this dependence, we found that knockdown of Hsp70 in certain tumor epithelial cell lines can cause senescence. Senescence is an irreversible growth arrest with specific cell morphology (e.g., enlargement and flattening) and biochemistry (e.g., senescence-associated secretory phenotype and expression of SA- β -galactosidase)^{51, 52}. While apoptotic cells commit suicide, senescent cells stay alive for a

long time *in vitro*, but they can be effectively eliminated *in vivo* by the innate immune system⁵³. Senescence is considered to evolve as an intrinsic mechanism to prevent propagation of cells with unrepaired DNA or cells that express major oncogenes (oncogene-induced senescence, OIS)⁵⁴. We hypothesized that Hsp70, being upregulated in tumor cells, may allow cells to bypass the OIS barrier⁵⁵. This idea was based on the observation that whereas knockdown of Hsp70 in normal human mammary epithelial cells does not affect their growth, it causes senescence in the cells transformed with RAS, Her2, or PIK3CA oncogenes (Fig. 1)⁵⁵.

The bypass of OIS modulated by Hsp70 in transformed cells may involve the p53-p21 pathway. Indeed, in tumor cells with wild type p53, Hsp70 knockdown stabilized p53 and increased p21 levels, while in cells with p53 mutation there was no increase in p21⁵⁶. Since p53 is frequently mutated in a variety of tumor types, it is possible that disabling p53-mediated senescence may release tumors from their Hsp70 dependence. However, even tumors with mutant p53 retain their dependence on Hsp70 because of the p53-independent pathways of senescence⁵⁵. Our finding suggested that these alternative senescence pathways activated upon Hsp70 depletion in cancer cells with mutant p53 could associate with decrease in the cell cycle regulator survivin or activation of the ERK pathway⁵⁵.

Hsp70 is critical for cancer development

Following correlative human population studies and cell culture experiments, the ultimate question remained whether Hsp70 is mechanistically involved in tumor development. Recent studies with animal cancer models provided experimental evidence in favor of this possibility. For example, expression Her2 (NeuT) oncogene in mammary epithelial cells triggered invasive breast cancer with 100% penetrance in control animals, while in Hsp70 knockout mice expression of Her2 rarely led to development of cancer⁵⁷. Lack of tumors in the knockout animals indicated that Hsp70 is essential for early stages of tumor development, consistent with its role in suppression of the oncogene-induced senescence. Further supporting this notion was the finding that Hsp70 knockout suppressed hyperplasia of mammary epithelium induced by Her2, which is normally seen in young mice long before tumor development. Furthermore, ducts and alveoli in Her2-expressing Hsp70 knockout mice were filled with senescent cells, while without Her2 expression these mice had normal mammary tissue⁵⁷. This experiment indicated that endogenous Hsp70 prevents OIS in the model of Her2-positive breast cancer and thus allows tumor emergence.

The role of Hsp70 in regulation of OIS was further corroborated by a recent publication that did not find significant effects of Hsp70 knockout on either survival or tumor emergence in a model that combined Ras and E6 oncogenes⁵⁸. In fact, E6 downregulates p53 and effectively reduces expression of its downstream target p21⁵⁹. Accordingly OIS was also suppressed and could not be triggered by the Hsp70 knockout in this model, and therefore such a knockout could not inhibit tumor emergence. Similarly in polyoma middle T antigen (PyMT)-induced breast cancer model, in which minimal OIS was seen⁶⁰, Hsp70 knockout did not reduce tumor emergence (Gong, personal communication).

Since bypassing OIS is critical during the early steps of tumor initiation, Hsp70 should be seen at elevated levels in low grade tumors. While this has been reported, curiously, the levels of Hsp70 increase further in high grade cancers^{15, 61} suggesting that Hsp70 may play distinct role(s) in later stages of cancer progression that may require even higher levels of the chaperone. Since in a model of Her2-positive breast cancer Hsp70 is essential for tumor initiation, to assess the effect on metastasis, a distinct breast cancer model lacking OIS must be used. Indeed in the PyMT model that shows little OIS, Hsp70 knockout dramatically suppressed metastasis (Gong et al., submitted). Two independent factors appear to contribute to the effects of the Hsp70 knockout. First, the lack of Hsp70 leads to a depletion of cancer stem cells, and tumors emerging in PyMT/hsp70^{-/-} mice are poorly transplanted into other host mice. Second, the overall population of tumor cells demonstrates reduced motility and invasion (Gong et al., submitted). These genetics data indicate that Hsp70 is critical for both tumor initiation and metastasis.

The problems with “non-oncogene addiction” hypothesis

Recent studies on sequencing the genomes of diverse human tumors did not reveal mutations or amplifications of Hsp70 (though certain SNPs in the Hsp70 gene HspA1A show association with cancer^{62,63,64}). Thus, these observations imply that regulation of Hsp70 expressions should occur mainly at transcriptional and/or translational levels. Accordingly, data from the human cancer microarrays database Oncomine show that in many tumors Hsp70 mRNA levels are increased compared to corresponding normal tissues. This induction of Hsp70 may be associated with increase in levels and activity of the heat shock transcription factor Hsf1, the master regulator of transcription of heat shock proteins, which is also overexpressed in many cancers⁶⁵. In line with these considerations various noxious factors of tumor microenvironment, like hypoxia or lack of energy, can cause proteotoxic stress, activate Hsf1, and induce Hsp70 (see ref⁶⁶ for review). For example, during progressive growth of ascites tumor cells *in vivo*, which associates with development of hypoxia and decrease in ATP, we observed increased levels of major Hsps, including Hsp70⁶⁷). However, the idea that proteotoxic stress in cancer can be responsible for upregulation of Hsp70, being plausible during *in vivo* tumor growth, cannot explain why tumor cell lines cultured without a stress *in vitro* also have elevated levels of Hsp70.

An attractive hypothesis of “non-oncogene addiction” has been proposed a number of years ago to explain the dependence of cancer on high levels of chaperones⁶⁸. According to this idea, cancer cells, which are often aneuploid, have a disbalance in protein complexes, and therefore increased demand for molecular chaperones. In other words, cancer cells experience intrinsic proteotoxic stress which leads to permanent activation of Hsf1 that enhances expression of chaperones. Such permanent proteotoxic stress leads to “addiction” of cancer cells to Hsf1, Hsp70 and other Hsps, which become essential for their survival. Thus, it was suggested that Hsp70, not being an oncogene by itself, is essential because it prevents an intrinsic proteotoxicity in tumor cells. This idea is widely accepted in the field and is supported by certain evidences. For example, aneuploid strains of yeast are more sensitive to conditions that interfere with protein translation, folding and degradation^{69,70}. Also, mammalian cell strains that have certain extra chromosomes demonstrate higher levels of Hsp70 and increased sensitivity to inhibitors of the chaperone Hsp90⁷¹.

The concept of the requirement for Hsp70 in cancer cells due to aneuploidy and associated increased demand for chaperones directly predicts that upon knockdown of Hsp70, cancer cells must experience proteotoxic stress. However, as we have recently found, depletion from cultured breast or cervix human cancer cells of the major chaperone Hsp70 did not generate proteotoxic stress⁷². As a measure of proteotoxic stress, several criteria were used in these experiments, including (a) levels of ubiquitinated proteins, (b) activation of Hsf1, (c) activation of Nrf2, a transcription factor that responds to oxidative and proteasome stress, and (d) luciferase refolding *in vivo*⁷². Similar data were reported with depletion of another major chaperone the member of the small heat shock protein family Hsp27⁷³. Importantly, upon depletion of either Hsp70 or Hsp27, a dramatic reduction of cell growth⁵⁶ and massive senescence were seen⁷³. As noted previously, these effects were specific to transformed cells and were not seen in normal breast epithelial MCF10A cells^{55,57}. These data directly indicate that higher levels of Hsp70 and “addiction” to this and other chaperones is unrelated to intrinsic proteotoxicity in cancer cells and must have a different underlying mechanism, e.g. regulation of cancer cell signaling (see next section).

What could be the mechanisms of chaperone induction in tumor cells beside intrinsic proteotoxic stress? Recent data demonstrate that such induction could be directly associated with oncogene signaling. For example, in early studies it was demonstrated that the major oncogene c-myc can induce transcription of Hsp70⁷⁴. Induction of Hsp70 was also seen upon expression of RET oncogene involved in thyroid cancer⁷⁵ and BCR-Abl responsible for chronic myelogenous leukemia⁷⁶. Similarly, the Her2 oncogene can activate Hsf1 and induce Hsp70⁵⁷. Interestingly, heregulin, a ligand of related tyrosine kinase receptors ErbB1 and ErbB3, can also activate Hsf1⁷⁷. Phosphorylation of Hsf1 by mTOR, a downstream component of the ErbB pathway, was found to activate Hsf1, indicating a direct role for signaling (rather than proteotoxicity) in Hsf1 regulation⁷⁸. The mTOR pathway is also frequently upregulated in cancer by oncogenic PIK3CA and PTEN mutations⁷⁹, which may also contribute to Hsf1 activity. Of note, it was shown that mutations in the p53 tumor suppressor also lead to activation of Hsf1 via the EGFR/ErbB2 pathway, suggesting that this pathway may integrate various signals to regulate Hsf1, and subsequently expression of Hsp70⁸⁰. It would be interesting to test whether these *in vitro* results on the role of oncogene signaling have relevance to human tumors, considering that data regarding expression of oncogenes and Hsp70 in a variety of human cancers became recently available.

Hsp70 regulates multiple signaling pathways

Original findings that Hsp70 suppresses apoptosis and oncogene-induced senescence suggested that it can interfere with signaling pathways that govern these processes. Indeed, a large number of publications from many labs indicated that Hsp70 affects cellular signaling pathways. For example, Hsp70 suppresses activation of multiple kinase cascades, including JNK, p38 and ERK^{37,38,81}. Via regulation of these cascades Hsp70 influences many physiological and pathological processes, like inflammatory processes²⁷⁻²⁹ or development of type II diabetes⁸². Several cancer-related signaling pathways regulated by Hsp70 have been reported beside above-mentioned JNK and ERK. For example, Hsp70 was found to control expression of the major regulator of OIS, the cell cycle inhibitor p21⁵⁶, which is

critical for establishing senescence upon Hsp70 depletion⁵⁶ (Fig. 2). In part, increase in p21 levels resulted from stabilization of p53, which in turn was mediated by effects of Hsp70 on the E3 ligase Mdm2⁵⁶. On the other hand, Hsp70 could also exert its effects on p21 in a p53-independent manner, which involves the transcription factor FoxM1⁸³ (Fig. 2). Overall, these pathways modulate effects of Hsp70 on OIS, and thus control the process of cancer initiation.

In relation to cancer progression and metastasis, Hsp70 was shown to regulate a number of pathways, including transcription factors Hif1 α , NF- κ B⁸³(Fig. 2). Effects of Hsp70 on FoxM1 also contribute to cancer progression and metastasis⁸³. In addition, depletion of Hsp70 causes downregulation of a major translation modulator HuR, which controls expression of multiple proteins involved in tumor growth, invasion and metastasis⁸³. These findings most likely represent just a tip of the iceberg, since Gene Set Enrichment analysis of microarray data suggest effects of Hsp70 on a large number of signaling pathways, the majority of which yet to be validated (Colvin et al, unpublished data). Therefore, Hsp70 appears to control a complex network of pathways that regulate multiple steps in cancer development both at transcriptional and translational level.

What are the mechanism(s) by which Hsp70 affects cell signaling? One mode of Hsp70 action appears to involve its interaction with Hsp90 and other co-chaperones. This complex is involved in stabilization of a multitude of client proteins, many of which are components of major signaling pathways, e.g. c-Raf or Akt⁸⁴. It was demonstrated that upon inhibition of Hsp90, it dissociates from the complexes, and Hsp70 (or cognate Hsc73) links the clients with E3 ligases, like CHIP, thus promoting their ubiquitination and. It was reported that depletion of Hsp70 together with the cognate Hsc73 mimics effects of inhibition of Hsp90 on degradation of multiple client proteins⁸⁵. The mechanistic basis for these effects is unclear since according to common paradigm Hsp70/Hsc73 is necessary for degradation of clients, and therefore its depletion should stabilize them, while the report demonstrated the opposite. In line with this observation, a recently developed inhibitor YK5, which targets a novel allosteric site on Hsp70 and Hsc73, had similar effects, indicating that involvement in degradation of Hsp90 clients significantly contributes to effects of Hsp70 on cancer⁸⁶. On the other hand, unlike double Hsp70/Hsc73 depletion, depletion of Hsp70 alone does not trigger downregulation of Hsp90 clients (our unpublished data), and therefore knockdown of Hsp70 alone on signaling pathways must be based on a distinct mechanism.

Mechanistically important, deletion of C-terminal EEVD sequence, which completely inhibited the chaperone activity of Hsp70, did not affect its function in suppression of JNK pathway and protection from TNF-induced apoptosis⁴². Similarly, with regulation of Raf-1 kinase, the chaperone activity of Hsp70 was dispensable⁸⁷. These data indicated that Hsp70 affects various signaling pathways in a mode that does not involve the chaperone function either in folding or degradation.

Bag3 mediates effects of Hsp70 on signaling

In search for a potential mediator of effects of Hsp70 on signaling, we turned to a co-chaperone Bag3, a unique member of the Bag-protein family. Beside the Bag region that can

interact with a motif in the ATPase domain of Hsp70 proteins, Bag3 also contains functional PxxP and WW domains⁸⁸ (Fig. 3), which may connect it to SH3 domains and PPxY motifs of signaling proteins^{89,90}. Notably, Bag3 has been implicated in tumor development⁹¹. Briefly, expression of Bag3 is upregulated in a number of cancers such as glioblastoma⁹², melanoma^{93,94}, pancreatic adenocarcinoma^{95,96}, and hepatocellular carcinoma (HCC)⁹⁷. Furthermore, overexpression of Bag3 correlates with dismal prognosis in melanoma⁹³, pancreatic carcinoma and HCC⁹⁷. Moreover, its serum level was proposed to serve as a biomarker of pancreatic adenocarcinoma⁹⁸.

Like Hsp70, Bag3 inhibits apoptosis by interfering with cytochrome c release, apoptosome assembly and other events in the cellular death program⁹⁹. Moreover, it takes part in the processes of cancer cell adhesion and migration^{91,100}. In human cancer cells, including lymphocytic and myeloblastic leukemic cells, Bag3 promotes cell survival and enhances resistance to chemotherapy^{101,102}. Importantly, expression of Bag3 is co-regulated with Hsp70 in Hsf1-dependent manner¹⁰³. Based on these functional data together with structural analysis of Bag3, we proposed that this factor may serve as a scaffold/mediator that transmits effects of Hsp70 to multiple signaling pathways that control cancer development.

So far, the main focus in studies of Bag3 has been on its role in autophagy and aggresome formation, two major protective responses to protein aggregation. Two models have been proposed to explain the requirement of Bag3 and Hsp70 for these processes. According to one model, Bag3 is physically involved in recruitment of aggregated proteins to microtubules for further transport to aggresome or formation of autophagic vacuoles^{104,105}. There is a significant circumstantial evidence to support this model; however, it has not been proven directly. According to another model, Bag3 transmits signals that regulate autophagy by mechanical stress, in part by binding to the LATS1 kinase via the WW domain to affect Hippo signaling pathway¹⁰⁶. These finding also implicate Bag3 in cancer signaling since Hippo pathway plays a major role in cancer cell stemness and epithelial-mesenchymal transition^{107,108}. Another cancer-related signaling factor regulated by Bag3 is PLC γ ¹⁰⁹. Bag3 interacts with the SH3 domain of this protein via its PXXP region¹⁰⁹ (Fig. 3). While implicating Bag3 in cancer signaling, these data also suggested that Bag3 can serve as a direct mediator of effects of Hsp70 on cancer, and our recent work addressed this possibility.

Using several cell lines, we showed that depletion of Bag3 has similar effects on multiple signaling pathways as depletion of Hsp70. Indeed, knockdown of either Bag3 or Hsp70 downregulated FoxM1 and survivin and upregulated p21 and p27, and both depletions suppressed Hif1 and HuR⁸³ (Fig. 2) Importantly, overexpression of Bag3 mutants with substitutions in Bag domain that cannot interact with Hsp70 mimicked effects of depletions, indicating that Hsp70-Bag3 interaction is critical for regulation of these pathways⁸³. Interestingly, effects of Bag3 and Hsp70 knockdowns on NF- κ B differed significantly. Unlike Hsp70 depletion, Bag3 depletion did not activate NF- κ B, but at the same time it prevented activation of NF- κ B by depletion of Hsp70⁸³. In other words, Bag3 was critical for regulation of NF- κ B by Hsp70. Taken together, it appears that a network of cancer

signaling pathways is regulated by Bag3-Hsp70 module and the effects may vary mechanistically with different pathways.

Direct biochemical test whether Bag3 mediates effects of Hsp70 on signaling pathways came from investigation of Src. As with NF- κ B, Hsp70 regulates Src activity in a Bag3-dependent manner⁸³. Importantly, PXXP region of Bag3 directly interacts with the SH3 domain of Src, and this interaction requires association of Hsp70 with Bag domain⁸³ (Fig. 3).

In summary, Hsp70 is involved in regulating a complex signaling network that involves major cancer-related factors, including FoxM1, p21, p27, survivin, HuR, Hif1, NF- κ B, Src, as well as other pathways (s.2). When individually considered, these pathways could have cancer-promoting or cancer-repressing effects. Nevertheless, we know that at least in breast cancer models, knockout of Hsp70 suppresses both tumor initiation and progression, indicating that the net outcome of this complex Hsp70 signaling network to be pro-oncogenic.

The network of pathways regulated by Hsp70 may be even more complex since distinct Bag-family members may also mediate effects of Hsp70 on signaling. For example, originally it was reported that Bag1 mediates effects of Hsp70 on Raf1⁸⁷. Bag1 has ubiquitin-like domain that is involved in its interaction with various components of the ubiquitin-proteasome system, which increases putative complexity of the system¹¹⁰. In another example, Bag6 may also be involved in signaling since it has a proline-rich domain¹¹¹ which may also be involved in interaction with SH3-domain proteins.

Targeting Hsp70 for cancer treatment

Overall, concerted efforts of multiple labs uncovered that Hsp70 satisfies major requirements of an anti-cancer drug target. Indeed, (1) Hsp70 levels are strongly elevated in a large fraction of various cancers compared to normal corresponding tissues, (2) Hsp70 is essential for growth and survival of cancer but not normal cells, (3) Hsp70 knockout mice are viable, and therefore Hsp70 is dispensable for normal tissues and even organism development, (4) Hsp70 is “drugable”. Accordingly, significant efforts have been undertaken to develop Hsp70 inhibitors for cancer treatment (see recent review by Jason Gestwicki and colleagues^{112, 113}). These studies indicated that (a) Hsp70-Bag3 module can be targeted by small molecules and used for cancer treatment, and (b) Hsp70-Bag3 interaction is not the only mechanism that defines effects of Hsp70 on cancer, and its other activities and interactions with distinct factors should also be considered for cancer targeting.

First molecule that showed effect on interaction between Hsp70 and Bag-family proteins was PES, which was originally developed by the group of Andrei Gudkov as inhibitor of mitochondrial p53¹¹⁴. PES can bind to Hsp70/Hsc70 and, showed potent anti-cancer effects in cell culture and a transgenic model of myc-induced lymphoma development¹¹⁵ (Table 1). Recently the next generation of PES-related compound (PES-CI) with higher affinity was developed and demonstrated increased potency in this mouse model¹¹⁶. Interestingly, though PES interacts with the substrate-binding domain of Hsp70¹¹⁶, it

effectively inhibits interaction of Hsp70 with various co-factors that interact with the ATPase domain, including J-domain and Bag-domain proteins¹¹⁵. Therefore, it is possible that interference with the Hsp70-Bag3 interaction may be a significant contributor to the anti-cancer effects of these compounds.

Another series of compounds that affect the Hsp70-Bag3 module is YM-1 and its analogs (Table 1). This series of compounds was developed by the Gestwicki group in their screen for molecules that affect the ATPase activity of Hsp70¹¹⁷. YM-1 prevents the nucleotide exchange, and thus freezes Hsp70 in the ADP-bound state^{118, 119}. Interestingly, in such a state, Hsp70 tightly associates with its polypeptide substrates, which facilitates their ubiquitination by the Hsp70-associated E3 ligase CHIP, and further proteasome-dependent degradation¹¹⁹. This property of YM-1 and related compounds could be used for treatment of neurodegenerative protein misfolding disorders, since administration of these compounds leads to rapid degradation of the disease proteins, e.g. tau¹²⁰ or androgen receptor with expanded polyglutamine domain¹¹⁹. Via stabilization of Hsp70 in the ADP-bound form, YM-1 can also potentially inhibit interaction of Hsp70 with Bag3 and other Bag-domain proteins both in a test tube and in cells⁸³. Because of this property of YM-1, we tested its effects on various signaling pathways controlled by Hsp70, including FoxM1, p21, p27, survivin, Hif1, HuR, and Src⁸³. YM-1 mimicked effects of Hsp70 depletion on these pathways both in cell culture and in a cancer xenograft mouse model⁸³. Furthermore, it selectively killed cancer cells in culture, and potentially inhibited cancer growth in mouse xenografts⁸³. Importantly, a close chemical analog of YM-1, which was significantly less potent in inhibition of the Hsp70-Bag3 interaction, did not affect these signaling pathways (manuscript in preparation), further suggesting that targeting the Hsp70-Bag3 module is responsible for the control of cancer-related signaling and anti-cancer activity of YM-1. Currently, the next generation of YM-1 analogs with higher potency and improved pharmacological properties is under development (Gestwicki, personal communication).

Though targeting Hsp70-Bag3 interaction appears to be most relevant to known cancer-promoting activities of Hsp70, its other functions may also contribute to cancer development and thus should be considered for targeting with small molecules. For example, there have been reports that Hsp70 is involved in chaperoning certain tumor suppressors, e.g. p53^{121–123} and therefore targeting the chaperone activity could be beneficial. Unfortunately, extremely strong binding to ATP makes it difficult to develop competitive inhibitors. Though one such inhibitor (VER-155008) has been developed by Vernalis¹²⁴ (Table 1), this company has abandoned the project because of the problems with further development and achieving reasonable specificity due to the similarity of the ATPase domain of Hsp70 to domains in actin and some other proteins. There were also attempts to target the chaperone activity by aptamers that bind substrate-binding or ATPase domains of Hsp70⁴¹. Such aptamers sensitized cancer cell to drug-induced apoptotic cell death in culture and in mice⁴¹(Table 1). On the other hand, because of the common delivery problems, the aptamers were expressed endogenously, and therefore their anti-cancer activity in a relevant cancer model has not been evaluated.

A distinct alternative approach has been to target interaction of Hsp70 with DnaJ co-chaperones. For example, a flavonoid myricetin was shown to bind to the ATPase domain of

Hsp70 and inhibit DnaJ binding¹²⁵. Unlike Bag-domain proteins that serve as nucleotide-exchange factors, DnaJ proteins facilitate interaction of Hsp70 with certain substrates and stimulate ATP hydrolysis¹²⁶. These proteins bind to a distinct surface on the ATPase domain of Hsp70, and accordingly myricetin that interferes with the Hsp70-DnaJ interaction¹²⁵ did not affect interaction between Hsp70 and Bag3. In line with this finding, myricetin did not affect signaling pathways regulated by the Hsp70-Bag3 module¹²⁵. Nevertheless, myricetin demonstrated potent anti-cancer activities¹¹⁷¹²⁷ (Table 1). However, as with other flavonoids, myricetin interacts with many proteins, e.g. thioredoxin reductase¹²⁸, DNA polymerase, topoisomerase I¹²⁹, and proteasome¹³⁰ which may define its anti-cancer effects (Table 1).

Another series of inhibitors of the Hsp70-J-domain interaction were developed using the screen for ATPase activity of Hsp70 stimulated by DnaJ¹³¹. A compound MAL3-101 potently inhibited the DnaJ-stimulated ATPase, but not the intrinsic ATPase activity. Though its effect on the Hsp70-Bag3 interaction has not been tested, this series of compounds is unlikely to affect such interaction because J-domains bind to the opposite side of the ATPase domain of Hsp70. MAL3-101 demonstrated potent activity against multiple myeloma and Merkel cell carcinoma both in cell culture and animal models¹³²¹³³ (Table 1), strongly suggesting that DnaJ-related activities of Hsp70 unrelated to regulation of signaling via Hsp70-Bag3 module may be important for cancer development. Overall these experiments indicate that targeting the chaperone activity of Hsp70 either by interference with binding to substrates, or ATP, or DnaJ co-chaperones may be useful for cancer treatment. Recently a new allosteric site was identified in the ATPase domain of Hsp70, and a small molecule that binds to this domain has been developed⁸⁶ (Table 1). This molecule YK5 was shown to affect function of Hsp70 in Hsp90 complexes. Conventional inhibitors of Hsp90 destabilize client proteins, including factors involved in cancer, and facilitate their degradation, which defines anti-cancer effects of such inhibitors¹³⁴. Unfortunately, alone with depletion of cancer factors, they activate Hsf1 and enhance transcription of Hsp70¹³⁵. In turn, induction of Hsp70 suppresses apoptotic signaling and protects cells from Hsp90 inhibitors, which jeopardizes their anti-cancer activity¹³⁶. Interestingly, while mimicking effects of Hsp90 inhibitors on degradation of Hsp90 client signaling proteins, YK5 did not trigger induction of Hsp70, and therefore it may eventually demonstrate a much better potency than Hsp90 inhibitors⁸⁶. Notably, YM-1 showed only a very minor effect on major Hsp90 clients, further indicating that its anti-cancer effect is distinct and related to inhibition of the Hsp70-Bag3 module (manuscript in preparation).

A critical question in development of Hsp70 inhibitors as drugs is their specificity towards members of the Hsp70 family. The ideal drug, of course, would target Hsp70 only, but not the cognate Hsc73 or other family members. Due to the structural similarity of Hsp70 protein family this goal appears unrealistic, and so far existing drugs do not show significant specificity towards Hsp70 over other family members. A recent discovery, however, suggests an interesting approach towards development of specific drugs. Gestwicki's group demonstrated that methylene blue is a mild oxidizer that can specifically oxidize a cysteine in the ATPase domain of Hsp70, and inhibit its ATPase activity¹³⁷. This cysteine residue is absent in Hsc73, thus providing a specificity of methylene blue against Hsp70. Though this molecule does not affect the Hsp70-Bag3 module (Gestwicki, personal communication), it

affects the chaperone activity, and therefore may target oncogene folding or stability thus exerting anti-cancer effects.

Overall Hsp70 has matured as an attractive target for drug design, and targeting Hsp70-Bag3 interaction or the chaperone activity of Hsp70 could prove to be highly beneficial.

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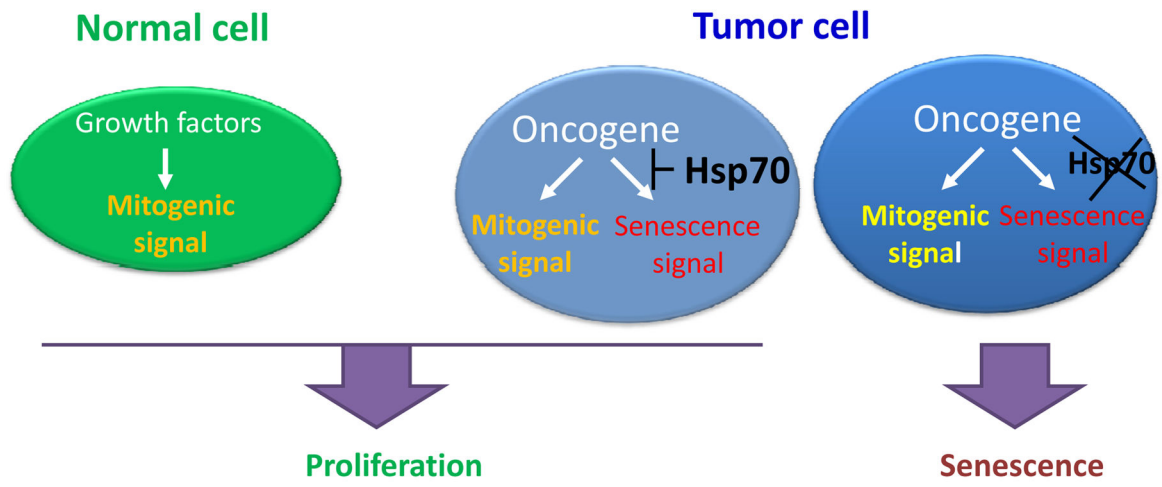


Fig. 1.
Tumor cells overexpress Hsp70 to prevent oncogene-induced senescence.

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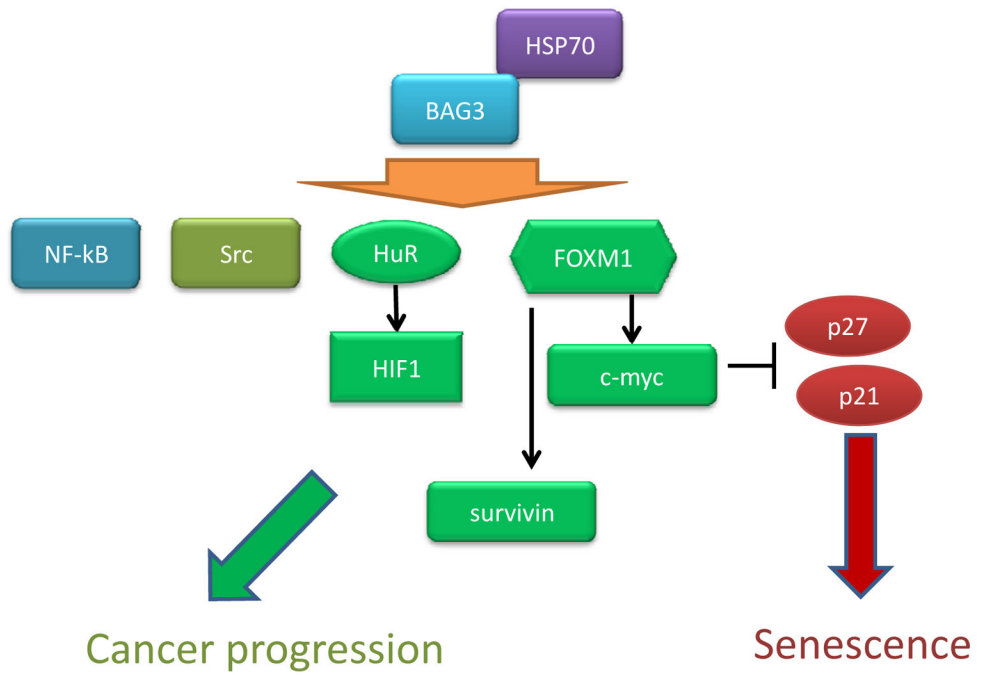


Fig. 2. Hsp70 regulates multiple pathways in cancer cells via interaction with BAG3 cochaperone.

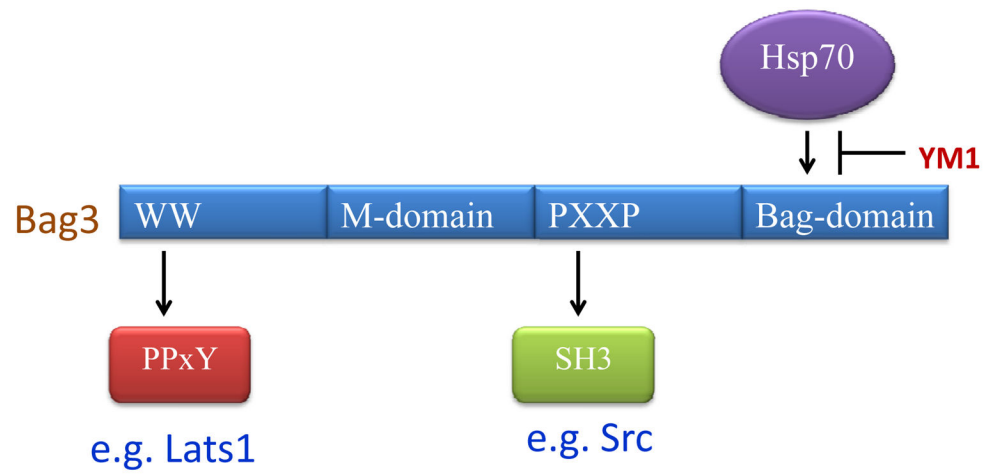


Fig. 3. Domain structure of Bag3 cochaperone and its involvement in cell signaling.

Table 1

Inhibitors of Hsp70

Name	Target	Pathway inhibited	Effect in vitro	Effect in vivo	Refs
Apoptomer	Hsp70 Substrate-binding, ATPase	Anti-apoptotic	Sensitization to drug-induced apoptosis	Inhibition of B16 melanoma growth	41
MAL3-101	DNAJ	Anti-apoptotic	Apoptosis, growth inhibition	Inhibition of multiple myeloma and Merccell cell carcinoma growth	132, 133
Myricetin	DNA J/Hsp(c)70	Proteasome, topo-I, thioredoxin reductase etc	Apoptosis	Inhibition of pancreatic cancer growth	125, 127, 128, 138
PES (pithifrin u)	Hsp(c)70	Autophagy	Growth inhibition	Delay in myc-induced lymphoma development	115
VER-155008	Hsp(c)70 ATPase	Hsp90 clients	Apoptosis, growth inhibition	ND	124
YM-1	Bag3-Hsp(c)70	FoxM1, HuR	Inhibition of growth	Inhibition of growth of breast carcinoma and B16 melanoma	117
YK5	Hsp(c)70-Hsp90	Hsp90 clients	Inhibition of growth, apoptosis	ND	86