Biochemical Society Transactions (2018) **46** 51–65
https://doi.org/10.1042/BST20170335

Review Article

Gene expression regulation by heat-shock proteins: the cardinal roles of HSF1 and Hsp90

Gisela I. Mazaira[1], Cristina Daneri-Becerra[2], Nadia R. Zgajnar[2], Cecilia M. Lotufo[2] and Mario D. Galigniana[1,2]

[1]Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales de la Universidad de Buenos Aires, Buenos Aires, Argentina; [2]Instituto de Biología y Medicina Experimental (IBYME)-CONICET, Buenos Aires 1428, Argentina

Correspondence: Mario D. Galigniana (mgaligniana@conicet.gov.ar)

The ability to permit gene expression is managed by a set of relatively well known regulatory mechanisms. Nonetheless, this property can also be acquired during a life span as a consequence of environmental stimuli. Interestingly, some acquired information can be passed to the next generation of individuals without modifying gene information, but instead by the manner in which cells read and process such information. Molecular chaperones are classically related to the proper preservation of protein folding and anti-aggregation properties, but one of them, heat-shock protein 90 (Hsp90), is a refined sensor of protein function facilitating the biological activity of properly folded client proteins that already have a preserved tertiary structure. Interestingly, Hsp90 can also function as a critical switch able to regulate biological responses due to its association with key client proteins such as histone deacetylases or DNA methylases. Thus, a growing amount of evidence has connected the action of Hsp90 to post-translational modifications of soluble nuclear factors, DNA, and histones, which epigenetically affect gene expression upon the onset of an unfriendly environment. This response is commanded by the activation of the transcription factor heat-shock factor 1 (HSF1). Even though numerous stresses of diverse nature are known to trigger the stress response by activation of HSF1, it is still unknown whether there are different types of molecular sensors for each type of stimulus. In the present review, we will discuss various aspects of the regulatory action of HSF1 and Hsp90 on transcriptional regulation, and how this regulation may affect genetic assimilation mechanisms and the health of individuals.

General concepts

Molecular chaperones

The term *molecular chaperone* was first coined to describe the ability of nucleoplasmin to prevent the aggregation of histones with DNA during the assembly of nucleosomes [1]. Subsequently, the word was also used for proteins able to mediate the post-translational assembly of heterocomplexes, protecting them from denaturation and aggregation. Thus, when organisms are exposed to harmful or extreme insults, their cells dramatically increase the expression of a family of proteins collectively known as molecular chaperones or, simply, stress proteins. They represent the foremost constituent of the proteostasis network that safeguards and shields the cell proteome from damage [2]. Although the general consensus dictates that a decline of this network is detrimental to cell and organism health, it is currently accepted that a controlled perturbation of the proteostasis network may also offer new therapeutic avenues against human diseases [3]. This perception was initially evaluated with great concern because of the evolutionary conservation of these proteins, as well as their abundance, ubiquitous expression, and central roles in various aspects of protein biogenesis. Nonetheless, the development of specific small molecule proteostasis regulators for neurodegenerative diseases and cancer has made that proposal a current reality (see [3] for an updated review).

Received: 30 July 2017
Revised: 21 October 2017
Accepted: 27 October 2017

Version of Record published:
22 December 2017

Biochemical Society Transactions (2018) **46** 51–65
https://doi.org/10.1042/BST20170335

The term *heat-shock protein* (HSP) stems from the original observation that heat stress greatly enhances the production of a specific set of proteins. Nonetheless, although all HSPs induced by high temperatures are classified as molecular chaperones, not all molecular chaperones are necessarily HSPs. In this sense, it is noteworthy to emphasize that only 20% of the human chaperone network (i.e. 33 genes over a total of 169 genes) is indeed heat inducible [4].

Molecular chaperones play essential roles in almost all cellular events by assisting unfolded or misfolded macromolecules, a situation that gives the cells sufficient time to repair or resynthesize those damaged factors. In addition to regulating the proper folding of proteins exposed to environmental injury, molecular chaperones are also related to soluble protein transport and the subcellular localization of several factors [5]. They also modulate transcription by epigenetic regulation of gene expression and, even more intriguingly, heritable modifications of the state of chromatin [6–8]. All of these effects are achieved by various means, such as affecting the steady-states of nuclear factors in response to a number of physiological cues, by favoring the release of histones from the promoter regions of genes, and/or by altering the activity of epigenetic modifiers, for example histone deacetylases or DNA methyltransferases. Mostly based on findings in the cancer field, we are just starting to realize that both chemical post-translational modifications [9–12] and biochemical modifications due to co-chaperone and adapter protein recruitment [13–17] physically alter and modify the biological function of the interconnected network of molecular chaperones, not only by activation of the proteomic network, but also because this influences the subcellular localization of those chaperoned 'client' factors, as has been reported for a number of diseases [15,18–25].

HSF1 and the stress response

One of the major environmental factors established as an efficient inducer of epigenetic changes is stress, which is known to contribute to the pathogenesis of a number of disorders [26–28]. The stress response is a set of well-ordered and regulated reactions of the cell to stress, mainly characterized by high production of HSPs [29,30]. The induced HSPs protect cells, allowing their survival under conditions that would usually be lethal. Such a response can be triggered by a wide variety of causes beyond changes in the temperature (below or above the normal range), such as exposure to UV radiation, chemicals, toxic compounds, metals, inappropriate pH or osmotic pressure, nutrient starvation, oxidants, fever, cancer, infections and neurodegenerative diseases [31]. Also, the heat-shock response can be useful during the normal growth cycle of the cells, even without the existence of stressors in the medium.

The HSP family comprises five broadly conserved families of proteins classified according to their molecular weights: Hsp100, Hsp90, Hsp70, Hsp60, and the small HSP subfamily that includes Hsp33 and Hsp27 as signature members [25,31]. It is accepted that exposure of hydrophobic residues is the main mechanism by which HSPs distinguish a partially or globally unfolded protein from the properly folded native protein state [32].

In lower organisms such as yeasts, nematodes and *Drosophila*, the transcriptional activation of those genes encoding the HSPs is regulated by a single transcription factor, heat-shock factor 1 (HSF1). In contrast, in vertebrates, a family of four HSF members has evolved (called HSF1, HSF2, HSF3 and HSF4). HSF1 is the functional counterpart of the HSF1 in invertebrates. Accordingly, *hsf1*−/− mice are unable to increase HSP levels in response to thermal insult and show reduced survival skills upon stimulation with bacterial lipopolysaccharides [33]. Moreover, *hsf1*−/− fibroblasts exhibit no stress-induced expression of HSPs and they usually trigger the apoptosis program after being challenged by heat. Taken together, these observations suggest that in mammals, the function of HSF1 cannot be compensated for by the other members of the HSF family [34].

HSF1 shows a modular design (Figure 1A) composed of a heat-shock responsive element (HSE) DNA-binding domain, two heptad-repeat domains called HR-A and HR-B, a regulatory domain located downstream from HR-B (residues 201–330), a third heptad repeat sequence (HR-C), and the transcriptional activation domains. HSF1 is primarily cytoplasmic in its monomeric form, and is incapable of specifically binding DNA in the absence of a stimulus. It remains associated with the Hsp90-based heterocomplex, which includes the chaperones Hsp90 and Hsp70, and the co-chaperones p23 and the immunophilin FK506-binding protein 52 (FKBP52) [35] (Figure 1B). In this condition, HSF1 remains in its inactive form. The exposure of cells to stimuli such as heat-shock or chemical inducers, makes HSF1 homotrimerize, translocate to the nucleus, and acquire specific DNA-binding and transcription-enhancing activities [36]. The activity of HSF1 is regulated by oligomerization and transcriptional competence. Thus, heat treatment of cells promotes HSF1 binding to HSE DNA sequences, but this is insufficient for transcriptional activity [37]. Accordingly, some compounds are able to induce HSF1 homotrimerization, without enabling it to enhance the expression of HSP genes [38] and the

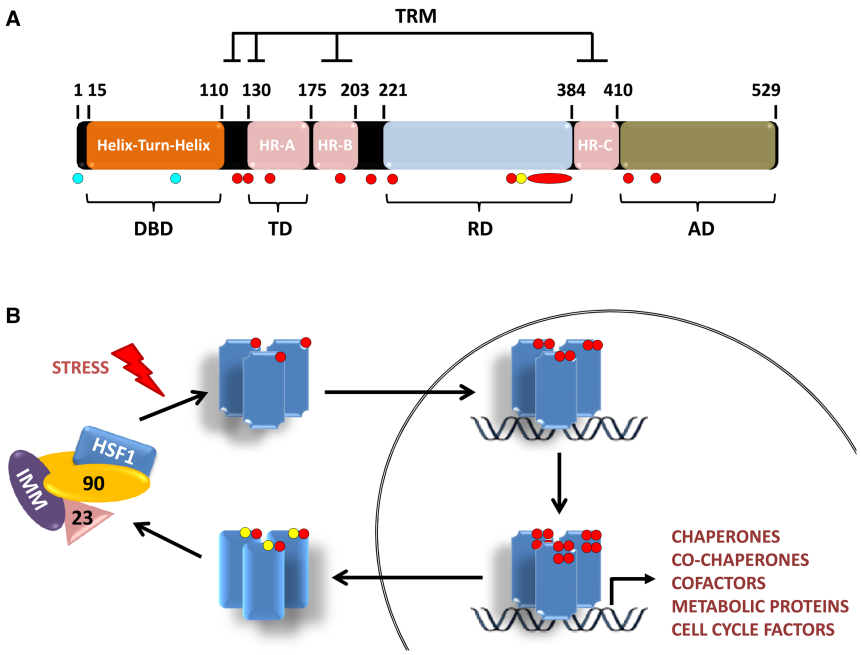

Figure 1. Structure and mechanism of activation of HSF1.

(**A**) Domain structure of human HSF1. AD, trans-activation domain; DBD, DNA-binding domain; HR, heptad repeat (leucine-zipper-like motif); RD, regulatory domain; TD, trimerization domain; TRM, trimerization repression motifs. Post-translational modification sites are shown as colored spheres: light blue represents acetylation (^{1}M, ^{80}K), yellow represents sumoylation (^{298}K), and red represents phosphorylations (^{121}S, ^{127}S, ^{142}S, ^{195}S, ^{216}S, ^{230}S, ^{292}S, ^{303}S, ^{307}S, ^{314}S, ^{319}S, ^{320}S, ^{323}T, ^{326}S, ^{344}S, ^{363}S, ^{367}T, ^{368}S, ^{369}T, ^{419}S, ^{444}S) [92]. (**B**) Mechanism of activation. Cytoplasmic HSF1 exists associated with a dimer of Hsp90, the co-chaperone p23 and a TPR-domain immunophilin (IMM) such as FKBP52. Upon the onset of stress, the chaperone complex is released, phospho-HSF1 homotrimerizes and translocates to the nucleus. Currently, it is unknown whether phosphorylation is a requirement for chaperone dissociation and where (cytoplasm or nucleoplasm) this process takes place. HSF1 is hyperphosphorylated and becomes transcriptionally active. Sumoylation favors DNA dissociation and consequently, the nuclear export of HSF1, which is reassembled in the cytoplasm as a monomer with the Hsp90-based chaperone complex.

overexpression of HSF1 results in accumulation of the trimeric form of HSF1 that shows negligible transcriptional activity [39]. Phosphorylation, sumoylation and acetylation mechanisms are also involved [40]. Even though the pool of trimeric HSF1 accumulated in cells overexpressing the factor binds to DNA, it is weakly phosphorylated and shows only marginal transcriptional activity. If these cells are exposed to calyculin A, a protein phosphatase inhibitor [41], both HSF1 phosphorylation and transcriptional activity are favored [42]. Interestingly, the regulatory domain of HSF1 is *per se* capable of conferring stress-sensitive repression of transcriptional competence on a chimeric factor [43]. It is sufficient to sense stress, and this is where the Hsp90–p23–FKBP52 chaperone heterocomplex binds, preventing the acquisition of transcriptional competence.

To date, how stressing situations are sensed by the cell and conveyed to HSF1 is not entirely understood. In view of the fact that a great number of stresses of varied nature can trigger a heat-shock response, one of the still unanswered questions is whether there are different types of molecular sensors for each stimulus. Another option could be that a proteotoxic environment may converge in a common factor or signal able to initiate the heat-shock response cascade of events. Figure 2 summarizes some of the proposed mechanisms of activation.

The classical model frequently described in the literature (Figure 2A) proposes that under normal conditions, HSF1 remains inactive when it is associated to molecular chaperones, most prominently the multitasking chaperone Hsp90. When cells are subjected to heat shock or other proteotoxic situations, the increased level of protein misfolding releases Hsp90 from HSF1 monomers allowing its trimerization [35,44]. Chaperone dissociation from HSF1 can be seen in cells microinjected with denatured, but not native, proteins [45]. Importantly, pharmacological inhibition with the Hsp90 inhibitor geldanamycin or reduction in the expression level of

Biochemical Society Transactions (2018) **46** 51–65
https://doi.org/10.1042/BST20170335

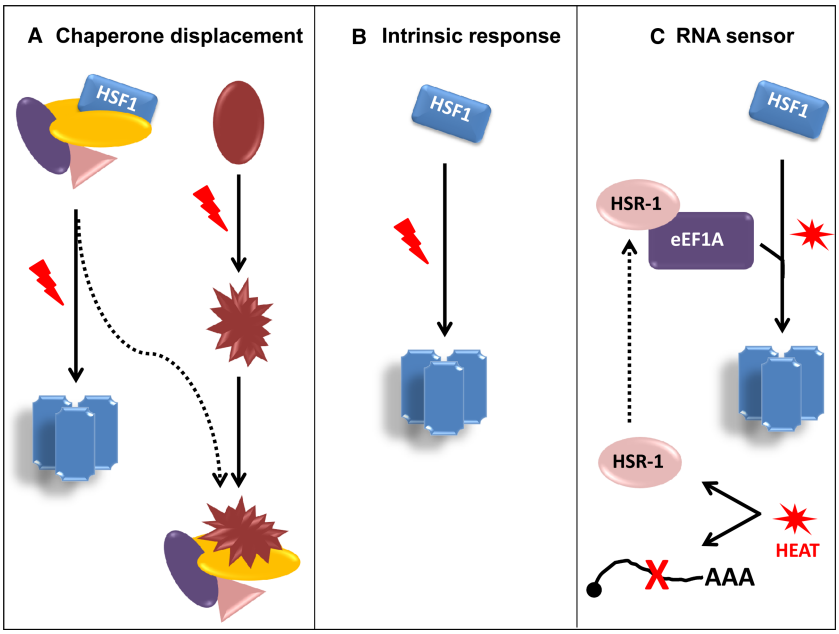

Figure 2. Models of HSF1 activation by different stimuli.

(**A**) Molecular chaperone displacement. In its inactive state, HSF1 is primarily cytoplasmic and remains associated with the Hsp90-based chaperone system. Upon the onset of stressing stimuli, for example high temperatures, the increased misfolding of other proteins favors Hsp90 release from HSF1, allowing HSF1 homotrimerization. (**B**) HSF1 intrinsic response. This model is based on the hypothesis that HSF1 itself possesses a built-in ability to detect proteotoxic stimuli. This is supported by experiments where purified HSF1 was converted into a homotrimeric oligomer by *in vitro* heat-shock and other stresses. (**C**) Thermic sensing by RNA. HSF1 activation is mediated by a complex between the heat-sensing RNA molecule HSR-1 and the elongation factor eEF1A, thereby linking HSF1 activation to the shutdown of translational processes during heat shock.

Hsp90, but not of Hsp70, heat-shock organizing protein (Hop), Hip, p23, CyP40, or Hsp40, dramatically activates HSF1 [46]. However the transcription of HSP genes is not affected [47]. This suggests a self-regulating step in the heat-shock response, where the HSPs may participate in the transcriptional machinery for the adjustment of *hsp* gene expression efficiency. In other words, the heat-shock response appears to be self-regulated at both transcriptional and post-transcriptional levels. During heat stress, binding of Hsp70 and Hsp40 inhibits HSF1 transactivating capacity (although it is less efficient than the abovementioned Hsp90•p23•FKBP52 mechanism), an effect perhaps related to the recruitment of the Hsp70-interacting transcriptional co-repressor CoREST [48]. This means that the HSF1–HSPs complexes generate a negative feedback mechanism to ensure that HSF1 activity is co-ordinated according to the overall condition of the protein-folding environment and the level of expression of its target genes. At this point, modulation by post-translational modifications of either protein cannot be ruled out.

Another interesting and perhaps parallel mechanism of regulation of HSF1 is its intrinsic ability to detect and respond to proteotoxic stimuli (Figure 2B). Thus, purified recombinant HSF1 becomes trimeric in a cell-free system by high temperature, low pH, oxidative stress or increasing concentrations of calcium (reviewed in [29]). The presence of two cysteine residues of the DNA-binding domain of HSF1 that are able to form a disulfide bond seems to be critical, since mutations of either residue impairs the heat-shock response [49,50]. Therefore, the built-in sensor capacity of HSF1 could also explain its fast activation during stress.

Nonetheless, it has also been observed that HSF1 binds to the Hsp70 promoter within a few seconds after the onset of stress, saturating the binding site in less than one minute [51,52]. Consequently, it has been proposed [52] that an alternative fast mechanism of activation of HSF1 by heat is also facilitated in the early stages by a ribonucleoprotein complex consisting of a translation elongation factor, elongation factor-1α (eEF1A), and heat-shock RNA-1 (HSR-1), a constitutively expressed non-coding RNA. According to this theory, called the RNA thermometer model (Figure 2C), HSF1 is activated by the heat-sensing RNA molecule HSR-1 complexed

with eEF1A, which leads to the overall translational shutdown observed during heat shock. The identification of this 'RNA temperature sensor' suggests that HSF1 activation by heat may be limited to temperatures that surpass the thermometer threshold.

Hsp90

Hsp90 is a ubiquitous molecular chaperone highly expressed in most organisms. Its expression is controlled by HSF1. Notably, HSF1 is a client of Hsp90 under normal conditions, being held in an inactive transcriptional complex along with Hsp70. Upon the onset of stress, Hsp90 is released and HSF1 monomers associate in a homotrimeric complex that acquires transcriptional activation. Thus, it is clearly implied that Hsp90 plays an important role in regulating its own transcriptional expression [53].

Figure 3A shows the three well characterized domains of Hsp90: (a) the highly conserved N-terminal domain (~25 kDa), where ATP and benzoquinone ansamycin inhibitors bind, (b) the middle domain (~40 kDa), which is the main area of the protein where client proteins interact, and (c) the C-terminal domain (~12 kDa), which is responsible for Hsp90 dimerization and interactions with tetratricopeptide repeat (TPR)-domain co-chaperones. The N-terminal domain and the middle domain are separated by a charged linker region also known as the 'connecting loop'.

Dimers of Hsp90 are in dynamic equilibrium between two states, the ADP-bound isoform and the ATP-bound isoform (Figure 3B). The ADP-bound Hsp90 shows higher affinity for the TPR-domain-containing co-chaperone Hop, which brings together Hsp70 and Hsp90. Hop binding to Hsp90 blocks ATP binding and the ATPase activity of the chaperone, which makes binding of the co-chaperone p23 significantly weak or void [46,47]. This ADP-bound conformation of Hsp90 is favored by the Hsp90-disrupting agent geldanamycin, a benzoquinone ansamycin antibiotic that shows high affinity for the nucleotide-binding site of the chaperone.

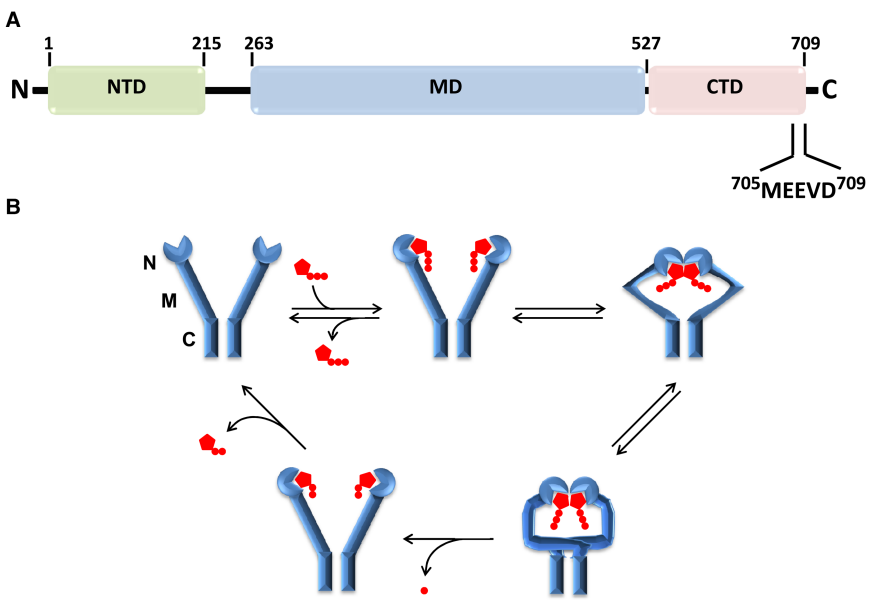

Figure 3. Heat-shock protein of 90 kDa.

(**A**) Structural domains. The N-terminal domain (NTD) is where both nucleotides ATP and ADP bind, and the binding domain for the specific Hsp90 inhibitor geldanamycin. The middle domain (MD) is also called the client-protein-binding domain and has a catalytic loop with an essential lysine residue that interacts with the Pγ of the ATP bound in the NTD. The C-terminal domain (CTD) has the dimerization site of Hsp90 and provides the MEEVD motif implicated in binding to co-chaperones with TPR domains. (**B**) Conformational cycle of Hsp90 dimers. The V-shaped open conformation of Hsp90 dimer binds ATP (depicted in red) at the N-terminal domain (N). The middle domain (M) repositions and interacts with the N-terminal domain (N), and the dimer twists over itself. The intrinsic ATPase activity is favored by this close association of the M and N domains, leading to the separation of the N-domains to release the products of the hydrolysis, and Hsp90 returns to the original open conformation to restart the cycle. The C-terminal domain (C) maintains Hsp90 as a dimer.

Biochemical Society Transactions (2018) **46** 51–65
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In this state, the associations of both p23 and TPR-domain immunophilins with the complex are impaired. On the other hand, when ADP is exchanged with ATP, the ATP-bound isoform of Hsp90 is stabilized by p23 binding, and other TPR proteins such as the high-molecular-weight immunophilins are efficiently recruited [48,49]. The ATP-bound isoform of Hsp90 is a better modulator of the biological activity of client proteins. Only the closed, N-terminally dimerized conformation can hydrolyze ATP.

As a result of gene duplication ∼500 million years ago, there are two Hsp90 isoforms that are 85% identical in mammalian cells, namely Hsp90α (the stress-inducible isoform) and Hsp90β (the constitutively expressed isoform) [29,54]. Hsp90 accounts for ∼2–3% of the total soluble proteins in resting cells, ∼6–7% in cancer cells, and up to 10% in highly stressed cells [30–34]. It exists as a homodimer mostly located in the cytoplasm, whereas a relatively small fraction of the Hsp90 population is nuclear (ranging from 2 to 3% of the total Hsp90 pool in resting cells [55]). Nonetheless, and under certain circumstances, Hsp90 accumulates in the nucleus [56,57] in an α/β-importin-dependent manner [58]. Actually, importin-β is a client protein of Hsp90 [35].

Hsp90 is also subject to a number of post-translational modifications, including phosphorylation [16], acetylation [36,37], *S*-nitrosylation [38], oxidation [39], ubiquitination [41], methylation [42] and tyrosine nitration [43]. Interestingly, histone deacetylase 6 (HDAC6) has been found to regulate the activity of Hsp90, such that inactivation or knockdown of HDAC6 leads to Hsp90 hyperacetylation, its dissociation from co-chaperone p23, and loss of chaperone activity. As a consequence, this prevents the maturation of the glucocorticoid receptor (GR), affecting ligand binding, nuclear translocation, and transcriptional activation [59,60]. Similarly, HDAC6 knockdown in prostate cancer cells also leads to Hsp90 hyperacetylation, impaired steroid-independent nuclear localization of the androgen receptor (AR) and the subsequent inhibition of prostate-specific antigen expression, and cell growth [61]. This shows that HDAC6 affects the transcriptional activity of other client proteins by regulating Hsp90.

Compared with other chaperone proteins, Hsp90 is highly selective for substrate recognition. The current consensus model is that Hsp90 provides chaperoning activity for client proteins that possess preserved tertiary structure. Rather than acting as a non-discriminant folding factor, the functions of Hsp90 in the cell seem to act preferentially as a delicate sensor of client protein regulation [7,44]. Most of what is known about the biological role of Hsp90 is due to examining steroid receptor folding, ligand binding, and nuclear translocation of these ligand-activated transcription factors. These studies established the critical co-operation of Hsp90 with other chaperones and co-chaperones, including Hsp70, Hsp40, p23, heat-shock organizing protein (Hop), and tetratricopeptide repeat (TPR)-domain immunophilins [7] (Figure 3A). These chaperones and co-chaperones function as a multiprotein machinery that makes vital contributions to the proper assembly and maturation of steroid receptors and protein kinases [7,45]. It is important to emphasize that when we refer to Hsp90, it is implied that many of these additional chaperones and co-chaperones are present in an Hsp90-associated oligomeric heterocomplex. Careful studies have demonstrated that the co-chaperone p23 serves to enhance Hsp90–client protein stability and, consequently, its biological function.

The TPR binding site of the Hsp90 dimer located in the C-terminal end recognizes proteins with degenerate 34 amino acid motives via the conserved pentapeptide MEEVD, located at the C-terminal end of Hsp90. TPR-domain proteins such as FKBP52, FKBP51, FKBP38, CyP40, XAP2, PP5, Hop, WISp39, Tom70, CHIP, and Tah1 bind to this TPR-acceptor site [50,51]. Hsp90 interacts with hundreds of client proteins and other signaling molecules, including steroid receptors, p53, tyrosine kinases, oncogenes, Stat5, NOS, HSF1, Akt, Chk1, Mdm2, IκB kinases, cytoskeletal proteins, importins, nucleoporins, histones, DNA helicases, telomerase reverse transcriptase, DNA polymerases, SmyD methyltransferases, p300 acetyltransferase, HDAC6, Aha1, SGT1, and Cdc37. In other words, Hsp90 creates a broad scaffold platform for the regulation of signaling cascades, nuclear architecture and cell function [44,46,54].

In spite of the great number of HSP90 client proteins studied to date [17,52], we know surprisingly little about the molecular basis of client protein recognition, which stands in remarkable contrast with other molecular chaperones. Inasmuch as the diversity of client factors that rely on Hsp90 chaperoning function, it is implied that a defined recognition motif probably does not exist. However, other accessory proteins and particularly Hsp90-binding co-chaperones should play a key role in client recognition, although the most important questions still remain unanswered – How? What is the exact role of the Hsp90 intrinsic conformational flexibility for this protein–protein interaction?

Post-translational modifications affect gene expression

In eukaryotes, the core of chromatin structure is composed of nucleosomes, histone octamer units typically enfolded by 147 base pairs of DNA that adjust local chromatin compaction and accessibility. Histones are subjected to multiple post-translational modifications that alter intrinsic chromatin properties, and present or hinder binding modules for non-histone proteins that form chromatin-modifying complexes. DNA itself is also a target of modifications. Overall, all of these modifications constitute the epigenetic code that is involved in all cellular processes by affecting gene expression and contribute to differential protein expression in different cells or in the same cell during its life [62]. In fact, epigenetic silencing is one the most practical ways to turn genes off. There are three major systems that can contribute to gene silencing: DNA methylation, histone modifications, and RNA-associated silencing [62]. DNA methylation is a chemical process that adds a methyl group to DNA by one of the three DNA methylases [63]. It is highly specific and always occurs in a region where a cytosine nucleotide is located next to a guanine nucleotide linked by a phosphate; this is called a CpG site [62]. Inserting methyl groups changes the emergence and structure of DNA, which in turn modifies interactions of genes with the transcriptional machinery.

In 1983, it was found that tissue from patients with colorectal cancer had less DNA methylation than normal tissue from the same patients [64], this finding being the first to link epigenetic modifications with cancer. Because methylated genes are typically silent, loss of DNA methylation can cause abnormally high gene activation by altering the arrangement of chromatin. On the other hand, excessive methylation can antagonize the protective effects of tumor suppressor genes. Most CpG cytosines are methylated in mammals, and there are extensions of DNA near promoter regions containing high concentrations of CpG sites (referred to as CpG islands) that are free of methylation in normal cells. However, these CpG islands become excessively methylated in cancer cells, thereby causing genes not to be silenced to turn off. This abnormality is the trademark epigenetic change that occurs in tumors and happens early in the development of cancer [62,63,65].

Hypermethylation of CpG islands can cause tumors by repressing tumor-suppressor genes. All of these processes are affected by the proper activity of the molecular chaperone Hsp90. In basal conditions, less than 5% of the total amount of Hsp90 is located in the nucleus [66,67], its functions being both the regulation of the biological activity of several nuclear factors and shaping the architecture of the nucleus (and, consequently, affecting gene expression). Also, it should be pointed out that non-CpG methylation has been described in mammalian stem cells and appears to be enriched in pluripotent cell types compared with differentiated cell types [68]. Non-CpG methylation, mostly at CpA sites, accounts for 25% of all methylated cytosines in stem cells, and it is almost absent in mature cell lines. Interestingly, non-CpG methylation is particularly abundant in brain tissue.

Current evidence shows cross-talks between Hsp90 and chromatin modifiers that affect the demethylation/methylation balance, thus turning genes on and off by mechanisms such as relaxing histone tails and altering the extent to which transcription factors are able to gain access to the DNA. SmyD is a new family of four members of chromatin regulators related to histone methylation that has been characterized [26,69,70], in particular the SmyD3–Hsp90 complex, which shows the particular feature to interact with the α isoform of the chaperone. The overexpression of this complex has been related to the development and progression of colorectal, liver and breast cancers [66,71]. SmyD3 interacts the MEEVD C-terminal motif of Hsp90α, and the disruption of this protein–protein interface has been related to the inactivating mechanism of Wnt gene transcription [72].

SmyD methyltransferase activity appears to be regulated by autoinhibition via the intra- and inter-domain bending of its conserved Hsp90-interacting C-terminal domain, and it was suggested that the mechanism of SmyD activation is similar to the association of the Hsp90-binding co-chaperone PP5 frequently associated with steroid receptors [27,28,53].

Another methyltransferase regulated by Hsp90 is DNA-(cytosine-5)-methyltransferase 1 (DNMT1) [73]. The chaperone is required to stabilize this methyltransferase, whose activity is high in breast cancer cells. It is associated with the estrogen receptor α (ERα) promoter, silencing ERα expression due to hypermethylation of 5′-CpG sequences. Because nuclear HDAC1 deacetylates Hsp90 [74], its pharmacological inhibition favors the existence of hyperacetylated Hsp90, which dissociates from DNMT1 and targets the methyltransferase to proteasomal degradation [74].

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Hsp90 influences the phenotypic manifestation of genetic variation

Despite a small fraction of Hsp90 being nuclear, its chaperoning activity on several transcription factors, cofactors, histones, and chromatin itself results in its essentiality for processes related to gene expression and therefore morphological evolution. In view of the broad spectrum of protein–protein interactions of Hsp90, it is not surprising that it is essential for cell survival and has been conserved among species during the course of evolution. Recently, Hsp90 has been regarded as a key factor at the crossroads of genetics and epigenetics. Studies performed in an experimental model that uses the fruit fly *Drosophila melanogaster* have led to the proposal that Hsp90 is a capacitor for phenotypic variation and morphologic evolution [54] (Figure 4). When Hsp90 was inactivated by protein mutation or pharmacologically by the use of selective disrupting agents, the phenotypic variation of *Drosophila* species affected almost all adult structures. This surprising observation led to the hypothesis that *D. melanogaster* had accumulated hidden genetic variations whose phenotypic expression was somehow prevented by the regular expression of Hsp90 [29,54]. It was consequently inferred that when the function of Hsp90 is compromised, its buffering properties are disrupted to the point that previously hidden or repressed phenotypic variants can emerge [8,30,31]. In other words, the reservoir of Hsp90 can mask biological variation, such that when environmental stress depletes that reservoir, new traits may appear in some individuals.

These astonishing contemporary findings provided experimental evidence for what Waddington [32] had proposed more than 70 years ago – a theoretical mechanism of evolutionary change that he named 'genetic assimilation'. The concept supported the idea that environmental and/or genetic factors can disrupt the usually stable course of development in living organisms. Such disruptions may then cause phenotypic variations that allow a given population to persist in a new, relatively hostile environment until new mutations permit the assimilation of the most beneficial phenotypic variants by natural selection.

Since Hsp90 is highly conserved across all species, these phenotypic buffering properties of Hsp90 were also demonstrated in plant model species such as *Arabidopsis thaliana*. It was demonstrated that pharmacological disruption of Hsp90 with geldanamycin or radicicol resulted in unusual phenotypes within a given inbred line [33]. Like *Drosophila*, plants exposed to elevated growth temperatures generated a spectrum of unusual phenotypes in most inbred specimens, whereas the adaptation for growing in the dark (considered a typical instance of phenotypic plasticity) showed varying levels of Hsp90 dependence among inbred lines. One important observation was that some of the 'new' phenotypes (i.e. leaf morphology and pigment accumulation) were actually beneficial for plants in the new environment.

All of these observations support a model in which Hsp90 buffers phenotypic variation, transforming this molecular chaperone into an intracellular regulator of adaptation. In short, Hsp90 seems to be responding to the call of Waddington's seminal concept mentioned above. However, the exact mechanism for this peculiar effect of the chaperone remains unclear. How Hsp90 is able to influence this phenomenon, how a given living being is able to evolve after identifying and appraising the proper factors within a stressful environment, or how Hsp90 is able to judge the optimal reaction to a particular stressor have yet to be fully elucidated. One possible partial explanation is provided in the next section.

Tuning transcription control by Hsp90

Hsp90 functions as a hub regulator of gene expression for many transcription factors. Perhaps the first and best known example is the case of a family of ligand-activated transcription factors, the steroid receptors. Hsp90 controls the ligand-dependent nuclear translocation of these proteins via dynein motors [34,55], which are linked to the receptor via the Hsp90–FKBP52 heterocomplex [56,57]. Both the Hsp90 co-chaperone FKBP52 and dynein are recruited by the receptor heterocomplex upon steroid binding, whereas FKBP51, another Hsp90 co-chaperone highly homologous to FKBP52 and unable to bind dynein [58], is released. The disruption of Hsp90 function by specific inhibitors such as geldanamycin or radicicol increases the nuclear translocation half-time of cytoplasmic receptors by ten-fold [53,56,57]. A recent study has reported that Hsp90 also favors the hormone-independent nuclear localization of AR in prostate cancer cells [35] and thereby plays an essential role in the progression of prostate cancer. In hormone refractory or androgen-independent prostate cancer cells, a large pool of AR translocates into the nucleus even in the absence of steroid, leading to the transcriptional activation of target genes resulting in tumor growth, a process that is blocked by geldanamycin [36].

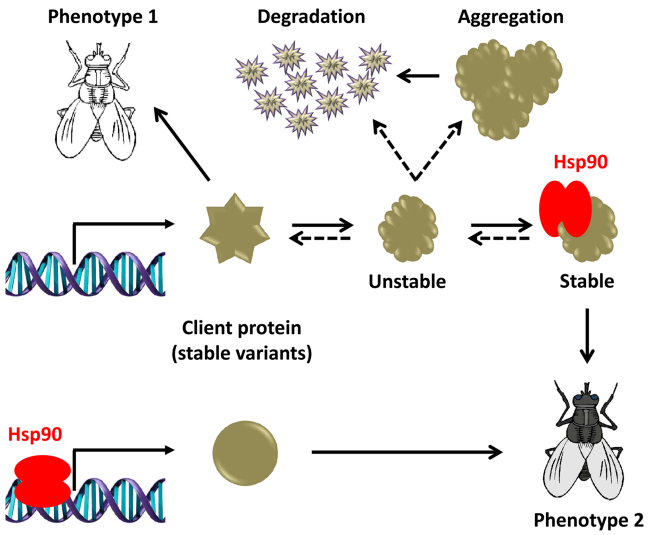

Figure 4. Hsp90-reliant mechanism of genetic assimilation.

Hsp90 can stabilize unusual active conformations of client proteins upon the onset of adverse environmental conditions, leading to a modification of its biological activity (for example, low protein kinase activity), which is in turn responsible for triggering unconventional signaling cascades that ultimately lead to the acquisition of a slightly different phenotype. Alternatively (but not in a mutually exclusive fashion), Hsp90 could favor the induction of a more stable protein variant for the novel environment conditions leading to the accumulation of an unusual 'isoform', whose modified activity also results in the generation of the same unconventional phenotype in a convergent manner with the above-postulated mechanism.

Additionally, the chaperone is instrumental in assembling multiprotein complexes such as RNA polymerase II (RNA pol II) in cytoplasm [37]. It was established that there is a functional link between the Hsp90 pool associated to chromatin and pausing of RNA pol II, and it was found that Hsp90 preferentially binds transcription start sites that exhibit RNA pol II pausing [37]. In this manner, Hsp90 controls the expression of a specific set of target genes by regulating paused RNA pol II at these loci. This molecular link is evolutionarily conserved and responds to environmental agents.

Upon the onset of stressing situations, most probably but not exclusively high temperature, a great number of heat-shock loci are targeted by HSF1 leading to their activation. Comparing the genome-wide distribution of HSF analyzed by ChIP-seq assays in *Drosophila* [38] for HSF-binding sites of heat-shocked cells with those of Hsp90 in normal cells [37] showed that approximately three-quarters of the 437 HSF targets found are occupied by Hsp90 in unstressed cells, suggesting a partnership between HSF and Hsp90 at chromatin. Given that HSF itself is an Hsp90-binding factor, the promoter-bound Hsp90 may assist HSF binding to nearby HSE sequences (or its activity thereafter). It could also be possible that HSF binds to many sites, but it activates genes only in the context of bound Hsp90 and paused polymerase. In line with this possibility, not all loci associated with HSF show the recruitment of elongation factors on polytene chromosomes [39].

The chaperone is also known to bind and regulate HIV promoter in infected cells [41], and has also been implicated in a signal-dependent disassembly of transcriptional regulatory complexes [42], activation of the histone methyltransferase SMYD3 [70], and nucleosomal removal [43]. Histones themselves are chaperoned by Hsp90 [44], which induces the condensation of the chromatin structure [45] and therefore may contribute to the tighter nuclear structure of heat-shocked cells, perhaps hindering the activity of several transcriptional factors. More recent evidence demonstrates that altered chromatin architecture impairs the response to heat shock [46] and may even influence emotional behavior [47].

Hsp90 has also been shown to interact and stabilize an important component of the epigenetic memory system in *Drosophila*, trithorax [48]. Hsp90 co-localizes with trithorax in *Drosophila* salivary glands, suggesting a direct role at the site of gene activity. Moreover, the chaperone is known to associate with and regulate HIV promoter in infected cells [41], stressing the need to investigate the general role of Hsp90 at transcription units in the genome. In addition, the activation of kinases related to chromatin remodeling also requires Hsp90, such

Biochemical Society Transactions (2018) 46 51–65
https://doi.org/10.1042/BST20170335

that key components of chromatin-remodeling complexes (RvB1/RvB2, Ino80, SWR-C and Tah1/Pih1, etc.) are also physically associated and functionally dependent on Hsp90 [12,49].

The HDCA6, Hsp90 and HSF1 connection

Histone deacetylase 6

The acetylation of histones and transcription factors is particularly relevant for the regulatory mechanisms of gene expression in that this post-translational modification affects chromatin architecture and, consequently, nuclear processes that could be passed to daughter cells as epigenetic markers. The balance between histone acetyltransferases and histone deacetylases controls a broader scope of basic cellular processes when it is compared with other major post-translational modifications and in many ways is comparable to phosphorylation [50–52].

Acetylation in lysine residues is a commonly occurring post-translational modification of histones and transcription factors. This is particularly relevant not only for the regulatory mechanism of gene expression in that it affects chromatin architecture and nuclear processes, but also because this property could be passed to daughter cells as epigenetic markers. Consequently, the balance between histone acetyltransferases and HDACs controls a more extensive number of elemental cellular events when it is compared with other types of post-translational modifications [50–52].

Among the 18 HDACs known to date, one of the most interesting is HDAC6 because it shows an exclusive property: it is located at the crossroads between cytoskeleton architecture and signaling cascades [75]. The α-tubulin subunit is a natural substrate of HDAC6 [76]. Overexpression of HDAC6 correlates with tumorigenesis and improved survival, whereas inhibition of HDAC6 promotes apoptosis [77]. Therefore, this acetylase may be used as a prognosis marker. HDAC6 function is modulated by Hsp90, which is in turn a substrate of the deacetylase [59,60], and it is required for the activation of HSF1.

It was first demonstrated that HDAC6 is primarily located in the cytoplasm thanks to a unique cytoplasmic anchoring domain with TPR motifs and a conserved nuclear export signal. Nevertheless, HDAC6 can accumulate in the nucleus during cell arrest in a similar manner as occurs in the presence of leptomycin B [78], a known CRM-1 inhibitor. HDAC6 is a substrate of p300 [79], which acetylates a lysine residue located at the N-terminal nuclear localization signal region. Mutation of this residue leads to a significantly reduced HDAC6 tubulin deacetylase activity and further impaired cell motility; however, the mutant has no effect on histone deacetylation. Interestingly, these mutations retain HDAC6 in the cytoplasm by blocking the interaction with importin-α [79]. Consequently, it may be possible that the acetylation status of HDAC6 may affect the degree of acetylation of histones. Accordingly, an important fraction of the nuclear pool of HDAC6 is complexed with HDAC11 [80], which could deacetylase HDAC6, stabilizing its nuclear pool. Importantly, in cancer cells a strong cytoplasmic localization of HDAC6 is observed, whereas a preferential nuclear localization is observed in normal cells [81].

HDAC6 and Hsp90

The fact that Hsp90 is a natural substrate of HDAC6 makes it a master modulator of cardinal nuclear processes such as transcriptional activity and chromatin architecture rearrangement. Acetylated Hsp90 loses affinity for the Hsp90 co-chaperone p23, which in turn destabilizes GR–Hsp90 complexes, impairing the glucocorticoid biological response because this ligand-activated transcription factor cannot be retrotransported to the nucleus [59,60]. The knockdown of HDAC6 in prostate cancer cells led to a predicted hyperacetylation of Hsp90 and, similar to those observations with the GR, AR nuclear localization is impaired, prostate-specific antigen expression is abolished, and cell growth is inhibited [61]. Similar representative examples of the importance of this post-translational modification of Hsp90 can be found in the stability properties of several nuclear factors, whose stability is usually increased by lysine acetylation, for example, p53, p73, Smad7, SREBP1a, SREBP2, Runx3, SF-1, ER81, FOXO4, NF-E4, HNF6 and E2F1 [82].

Another relevant property of the HDAC6–Hsp90 complex is to influence transcriptional regulation in its ability to form complexes with co-repressors. HDAC6 forms complexes with LCoR, a factor related to the ligand-dependent repressor activity of various nuclear receptors [83], as well as with ETO-2, a constituent of N-CoR, SMRT, and mSin3A complexes [84]. Other transcription factors able to interact with HDAC6, such as Runx2, also show individual activity as repressors. Thus, the functional repression of p21CIP/WAF has being assigned to a direct recruitment of HDAC6 to Runx2 from cytoplasm to chromatin [85]. The active subunits of

the transcription factor nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB) (p50 and p65/RelA) are able to interact with HDAC6 to repress the expression of the H^+/K^+-ATPase [86]. On the other hand, the knockdown of HDAC6 showed a slight action on dsRNA-induced activation of NF-κB, but it greatly impairs the activity of interferon regulatory factor 3 (IRF3), suggesting a potential dynamic role of the HDAC6–Hsp90 complex in the activation of IRF3 and the β-interferon gene response [87].

HDAC6 and HSF1

Targeting the proteasome or the aggresome to trigger the apoptosis program is one of the approaches to target cancer cells. Tumor cells accumulate misfolded proteins that are disposed by the proteasome and the aggresome [88]. These cells are consequently more reliant on the activity of the proteasome or the aggresome than non-malignant cells. Upon proteasome inhibition, the HDAC6 binding to those ubiquitinated proteins activates the aggresome pathway delivering these miniaggregates to dynein, moving them in a retrograde manner. Near the nucleus, intermediate filaments surround this particle and form aggresomes, which recruit chaperones and proteasomes activating the autophagic clearance. Thus, autophagosomes fuse to lysosomes, resulting in the degradation of the misfolded proteins [55]. The HDAC6 partner VCP/p97 (valosin-containing protein/protein 97) is a chaperone with segregase activity able to disassemble HDAC6 from the ubiquitinated protein aggregate. Thus, the ubiquitinated proteins are degraded via the proteasome instead of the aggresome [89].

In unstressed cells, VCP/p97 is recruited by HDAC6 to an Hsp90–HSF1 transcription factor heterocomplex to form a basal complex. In this manner, Hsp90 maintains the transcription factor HSF1 in an inactive state. Similarly, HDAC6 binds the ubiquitinated aggregates upon proteasome inhibition, and HDAC6 and Hsp90 dissociate from HSF1. On the other hand, the intrinsic ATPase activity of VCP/p97 appears to play a role in the dissociation of the Hsp90–HSF1 complex. Thus, it has been demonstrated that under proteasomal inhibition, HDAC6 interacts with polyubiquitinated proteins via its C-terminal region, which contains a zinc-finger ubiquitin domain [90], a phenomenon that is exclusive of the binding to VCP/p97, which dissociates from HDAC6 complexes. In turn, VCP/p97 dissociates the repressive HSF1–Hsp90 complex. After resorption of misfolded polyubiquitinated proteins when stress is over, VCP/p97 conversely recovers the free HDAC6, allowing the reassembly of the inactive HSF1 complex, suggesting that the key role played by HDAC6 in response to proteasome inhibition is multiple, thereby co-ordinating the formation of aggresomes and, through binding to ubiquitinated proteins, allowing the activation of HSF1 with the subsequent induction of the expression of HSP genes. In opposition to proteasome inhibition, the heat shock unexpectedly leads to HSF1 activation even in the absence of HDAC6. This apparent contradiction indicates that, unlike proteasome inhibition, heat-induced dissociation of the HSF1–Hsp90 complex is not dependent on both HDAC6 and VCP/p97 segregase activities. Therefore, the mechanisms underlying HSF1 activation depend on the nature of the stress. A recent study proposed that the differences observed in the early steps of HSF1 activation after subjecting cells to the two types of stress, heat shock and proteasome inhibition, can be explained by the duration of heat-shock response controlled by HDAC6 [71]. Thus, the induction of HSF1 by heat mobilizes the nuclear pool of HSF1 unrelated to the HDAC6–Hsp90 complex. In contrast, the dissociation of inactive HSF1 complex triggered by proteasome inhibitors primarily occurs in the cytoplasm, where HDAC6 is present associated with microtubules, and is a much slower process than the former. Also, heat should have a more profound effect on protein structure to affect the stability of the HSF1–Hsp90 complex in a VCP/p97- and HDAC6-independent manner. This could be one of the explanations we have currently for the above-mentioned conundrum related to the HSF1 regulatory mechanism. In spite of the large number of stresses of diverse nature able to trigger the stress response via HSF1, it is difficult to envisage whether there are different types of molecular sensors for each type of stimulus. Consequently, the development of new drugs targeting the ubiquitin-binding domain of HDAC6 could represent an interesting therapeutic strategy to down-regulate or prevent the accumulation of HSPs in cancer cells. In particular, it should be emphasized that both HDAC6 and HSF1 are able to regulate the Ras–Raf–MAPK signaling pathway, which is essential for the maintenance of the transformed phenotypes of cancer cells [72], Hsp90 being a key chaperone involved in all the steps of this oncogenic pathway [73].

Envoy

The biochemical property called epigenesis correlates with biological development and experience of a living being during its life span. It is an adaptation mechanism for adverse and potentially harmful situations. Individual differences in the vast majority of phenotypes probably reflect not only the effects of the unique

Biochemical Society Transactions (2018) **46** 51–65
https://doi.org/10.1042/BST20170335

experiences of that individual, but also the simultaneous impact of multiple sequence variants. The polygenic nature of various and intricate biological traits is likely to be reflected at the level of epigenetic scars.

At this point we have more questions than answers. It is unclear what type(s) of mechanism(s) prevail(s) under certain adverse situations and whether there is convergence of those mechanisms through multiple cell divisions. What is the temporal stability of regular DNA and protein marks and how do they correlate as a function of development or adaptation experience? It is uncertain whether our knowledge about specific regions of the genome to be modified may help to design more specific drug targeting, in particular if inter-individual variability is also considered. Do epigenetic modifications in the brain correlate with molecular fingerprints in peripheral tissues that can be used as markers of *in vivo* brain function?

The recent realization that the stress chaperone network is epigenetically distinct from its normal, housekeeping counterpart has opened the door for its selective targeting by small molecules. The classical chaperone function of cytosolic Hsp90 and several newly emerged moonlighting functions of Hsp90 at the nucleus leads to the hypothesis that the nuclear Hsp90 is structurally different from the cytosolic form due to post-translational modifications, whose specific identification could provide valuable information to determine cytosolic versus nuclear functions of Hsp90.

There have never been stronger efforts towards the identification of HSP modulators as in the past decade. Nevertheless, despite the numerous ligands discovered, we are still limited by the reduced number of chemical tools and lack of approved drugs. The situation is even more dramatic for the concert master of the stress response, HSF1. There are comparatively better results in the field of drug developing to target the epigenome. Current epigenetic pharmacological therapies provide clinical benefits through inhibiting DNMTs or HDACs. Three HDAC inhibitors, namely Zolinza (vorinostat), Istodax (romidepsin), and Beleodaq (belinostat), in addition to two DNMT inhibitors, Vidaza (azacitidine) and Dacogen (decitabine), have been approved by the FDA to date [74]. Several other HDAC and DNMT inhibitors are now in development. Other promising players include non-coding RNAs such as miRNAs and long RNAs that also are known to be important modifiers of the epigenome. In turn, these advances may also affect the treatment regimes in very specific pathologies, such as post-traumatic stress disorders. Actually, epigenetic actions of psychoactive drugs and psychoactivity of drugs affecting the epigenome have been already observed.

Recently, it was reported that prolonged treatment with Hsp90 inhibitors results in acquired resistance of cancer cells towards not only the assayed drug, but also to a broad spectrum of structurally distinct Hsp90 inhibitors [91]. Interestingly, this acquired resistance was inhibited by using HDAC inhibitors, supporting the idea that there is a potential benefit of using Hsp90 and HDAC inhibitors in combination within the clinical setting. Therefore, it could be hypothesized that the observed acquired pharmacologic resistance towards different types of Hsp90 inhibitors could be due to altered acetylation of Hsp90 or the result of HDAC action on the epigenetic regulation of gene expression. Nonetheless, to better assess the usefulness of 'epigenetic drugs', a better understanding of the exact epigenetic mechanisms is still needed, and particular attention needs to be directed towards the specificity and mechanisms of drug action, in addition to the time window of opportunity for treatment.

Abbreviations

AR, androgen receptor; DNMT1, DNA-(cytosine-5)-methyltransferase 1; eEF1A, elongation factor-1α; ER, estrogen receptor; FKBP, FK506-binding protein; GR, glucocorticoid receptor; HDAC, histone deacetylase; Hop, heat-shock organizing protein; HSE, heat-shock responsive element; HSF, heat-shock factor; HSP, heat-shock protein; HSR-1, heat-shock RNA-1; IRF3, interferon regulatory factor 3; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; PKC, protein kinase C; RNA pol II, RNA polymerase II; TPR, tetratricopeptide repeat; VCP/p97, valosin-containing protein/protein 97.

Funding

The authors acknowledge funding from the Agencia Nacional de Promoción Científica y Tecnológica de la Argentina, University of Buenos Aires, and Instituto Nacional del Cáncer.

Competing Interests

The Authors declare that there are no competing interests associated with the manuscript.

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