Post-translational modifications of Hsp90 and translating the chaperone code

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Cells have a remarkable ability to synthesize large amounts of protein in a very short period of time. Under these conditions, many hydrophobic surfaces on proteins may be transiently exposed, and the likelihood of deleterious interactions is quite high. To counter this threat to cell viability, molecular chaperones have evolved to help nascent polypeptides fold correctly and multimeric protein complexes assemble productively, while minimizing the danger of protein aggregation. Heat shock protein 90 (Hsp90) is an evolutionarily conserved molecular chaperone that is involved in the stability and activation of at least 300 proteins, also known as clients, under normal cellular conditions. The Hsp90 clients participate in the full breadth of cellular processes, including cell growth and cell cycle control, signal transduction, DNA repair, transcription, and many others. Hsp90 chaperone function is coupled to its ability to bind and hydrolyze ATP, which is tightly regulated both by co-chaperone proteins and post-translational modifications (PTMs). Many reported PTMs of Hsp90 alter chaperone function and consequently affect myriad cellular processes. Here, we review the contributions of PTMs, such as phosphorylation, acetylation, SUMOylation, methylation, *O*-GlcNAcylation, ubiquitination, and others, toward regulation of Hsp90 function. We also discuss how the Hsp90 modification state affects cellular sensitivity to Hsp90-targeted therapeutics that specifically bind and inhibit its chaperone activity. The ultimate challenge is to decipher the comprehensive and combinatorial array of PTMs that modulate Hsp90 chaperone function, a phenomenon termed the "chaperone code."

Molecular chaperones are necessary for the stability, folding, and activation of a wide array of "client proteins" (1). One such molecular chaperone, the 90-kDa heat shock protein 90 (Hsp90), has over 300 clients, including protein kinases, transcription factors, oncoproteins, and tumor suppressors. Tight regulation of Hsp90 chaperone function and the downstream activities of its client proteins is essential for the maintenance of proteostasis, execution of the full spectrum of normal cellular processes, and preservation of tissue and organismal health (2, 3). Hsp90 possesses an ATPase activity that is coupled to its chaperone function along with a series of Hsp90 conformational changes collectively known as the chaperone cycle (1). A group of proteins, called co-chaperones, and post-translational modifications (PTMs) of Hsp90 together regulate Hsp90 chaperone activity and fine-tune it to the needs of the client proteins and the cell (4–7). Cancer cells often use Hsp90 function to promote tumor growth and metastasis, proffering Hsp90 as an attractive therapeutic target (8–11). Specific Hsp90-targeted therapeutics can simultaneously affect a myriad of cellular processes through chaperone function inhibition. Despite strong preclinical evidence of Hsp90 inhibitor efficacy, several drugs have had only limited success in phase 3 clinical trials, and none has achieved FDA approval. Co-chaperone dynamics and Hsp90 PTM status contribute to the complex variables that determine Hsp90 inhibitor sensitivity (12–14). Effort toward elucidating the impact of post-translational modifications of Hsp90 on its chaperone function has found that these modification states alter Hsp90 ATPase activity, co-chaperone and client binding, client maturation, Hsp90 subcellular localization and degradation, and Hsp90 inhibitor sensitivity (15–17). Dozens of modification sites, including phosphorylation, acetylation, SUMOylation, methylation, and others, have been functionally and mechanistically studied, as will be reviewed here. Taken together, the literature suggests that we need to develop a holistic understanding of the combinatorial array of these PTMs that target Hsp90 and modulate its chaperone activity. This phenomenon is also known as the "chaperone code."

Structure and chaperone function of Hsp90

The two cytosolic isoforms of Hsp90, Hsp90α and Hsp90β, share 85% sequence identity (18–20). Hsp90β is constitutively expressed under normal physiological conditions. Hsp90α, however, is stress-inducible, and increased levels of Hsp90α have been associated with poor prognosis in cancer (20). The chaperone function of Hsp90 encompasses an ordered series of conformational changes coupled to its ATPase activity, collectively known as the "chaperone cycle" (Fig. 1) (6, 21–24). The functional unit of Hsp90 is a dimer, and each protomer consists of three structural domains (25–27). The amino-terminal domain, or "N-domain," contains the nucleotide-binding pocket, which also serves as the binding site for most Hsp90 inhibitors (28–30). The middle domain is connected to the N-domain by a flexible and highly charged linker region (31, 32). The middle domain harbors the catalytic loop that is necessary for ATP hydrolysis and also serves as the binding site for the majority of client proteins and many co-chaperones (1, 33, 34). The carboxyl-terminal domain, or "C-domain," is the site of constitutive dimerization of the Hsp90 protomers (35, 36) and contains the extreme C-terminal MEEVD sequence that serves as an

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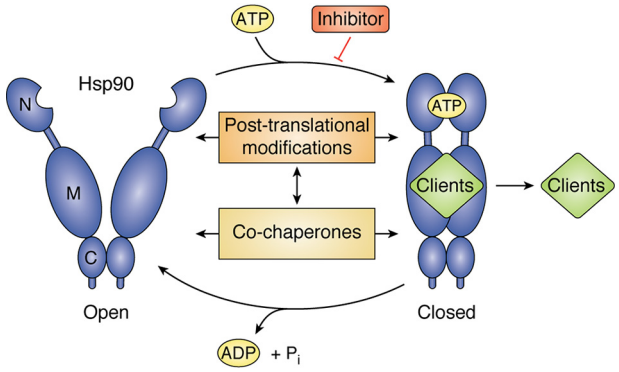

Figure 1. The Hsp90 chaperone cycle. Hsp90 begins its chaperone cycle in an open conformation that is dimerized only at the C-domain. ATP binding and an ordered series of conformational changes allow it to adopt a closed conformation, which is N-terminally dimerized. Upon ATP hydrolysis, Hsp90 returns back to the open conformation and is ready to begin another chaperone cycle. This allows for the activation of client proteins. This cycle is tightly regulated by co-chaperone proteins as well as PTMs, and Hsp90 inhibitors can also modulate the chaperone cycle.

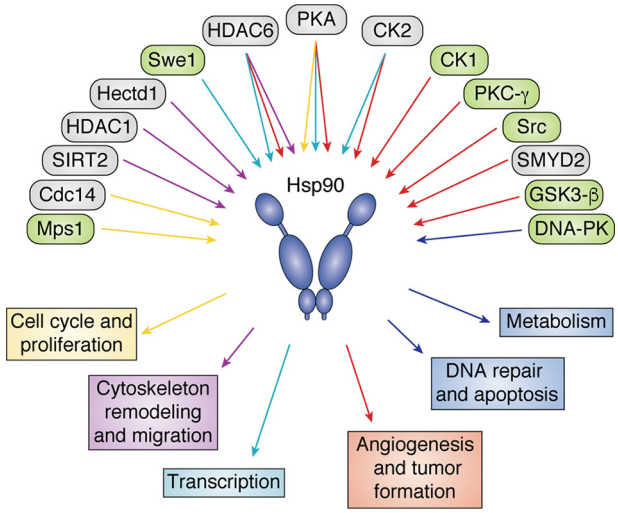

Figure 2. Modification by varied enzymes regulates Hsp90 function in biological processes. Enzymes known to modify Hsp90 regulate its PTM state, and this in turn influences various cellular functions as indicated. *Arrows* are *color-coded* to match the cellular functions. *Yellow arrows* indicate cell cycle and proliferation, *purple* indicates cytoskeleton remodeling and migration, *light blue* indicates transcription, *red* indicates angiogenesis and tumor formation, and *navy blue* indicates DNA repair, apoptosis, and metabolism. Enzymes that are also Hsp90 clients are *shaded* in *green*.

interaction site for tetratricopeptide repeat (TPR)-domain–containing co-chaperones (1). The Hsp90 chaperone cycle is considered to begin with an "open" dimer, which is only dimerized at the C-domain. ATP binding to the nucleotide pocket of the N-domain contributes to the large-scale conformational rearrangements that lead to transient dimerization of the Hsp90 N-domains. This transiently N-terminally dimerized state is referred to as the "closed" conformation. Upon ATP hydrolysis, Hsp90 returns to its "open" V-shaped conformation and is ready to begin another cycle (Fig. 1) (6, 24, 36–39). There is also a great deal of interdomain connectivity and communication across the Hsp90 protein (40–42). Progression through the chaperone cycle is required for client chaperoning and is tailored to individual client needs. In general, clients require chaperoning to either achieve a final active conformation, assemble into multiprotein complexes, or promote and stabilize a ligand-competent state that is awaiting activation (1).

Many factors contribute to Hsp90 chaperone cycle regulation and subsequent client chaperoning, including interacting co-chaperone proteins and post-translational modifications, and Hsp90 inhibitors can also affect the cycle. Co-chaperones, unlike client proteins, are not themselves dependent on Hsp90 chaperone function for their stability or activity. Generally, co-chaperones alter the progression of Hsp90 through the chaperone cycle by stabilizing different Hsp90 states and conformational intermediates. The TPR-containing co-chaperone Hsp70-Hsp90–organizing protein (HOP) binds to the open conformation of Hsp90, slows down its ATPase activity, and also helps transfer client proteins to Hsp90 (43–45). Client scaffolding or loading is another common attribute of Hsp90 co-chaperones. The co-chaperone cell division cycle 37 (Cdc37) specifically recruits kinase clients to Hsp90. The Cdc37-mediated chaperoning of kinases requires coordinated phosphorylation and subsequent dephosphorylation of Cdc37 by the phosphatase co-chaperone protein phosphatase 5 (PP5) (46–48). The co-chaperone activator of Hsp90 ATPase (Aha1), on the other hand, displaces HOP and helps to mediate Hsp90 N-domain dimerization and enhances the ATPase activity of Hsp90 (49–52). The co-chaperone prostaglandin E synthase 3 (p23) preferentially binds to and stabilizes Hsp90 in the closed conformation, slowing ATP hydrolysis and aiding client activation (26, 53). Co-chaperone dynamics, as well as several other processes, including Hsp90 ATPase activity, client activation, and Hsp90 inhibitor binding, are further altered by Hsp90 PTMs, as will be highlighted and discussed in the subsequent sections.

Post-translational modifications and Hsp90 function

Phosphorylation

Hsp90 is subject to various PTMs, as will be considered in the sections to follow. Phosphorylation of serine, threonine, and tyrosine residues is the most well-studied of these Hsp90 modifications. The earliest functional work demonstrated that treatment with the nonselective serine/threonine phosphatase inhibitor okadaic acid resulted in hyperphosphorylated Hsp90 and compromised chaperoning of the classic kinase client v-Src (54). As will be discussed, much of the functional work focuses on how different PTMs impact chaperone function via examination of ATPase activity of Hsp90, co-chaperone binding, and client stability. Additional works further detail how Hsp90 modification affects downstream cellular processes such as cell cycle control, DNA repair, and steroid hormone signaling as well as many others via effects on the stability and activity of Hsp90 client proteins (Fig. 2). Of note, a large number of potential Hsp90 modification sites in addition to those discussed below have been identified by high-throughput screening studies (RRID:SCR_001837). These studies will not be discussed here, as these sites have not been validated or linked to functional consequences; however, continued evaluation of these

sites is necessary to further our understanding and application of the chaperone code.

Serine/threonine phosphorylation

Chaperone cycle—As discussed above, Hsp90 chaperone function toward client maturation and downstream processes depends on the dynamics of the chaperone cycle, including Hsp90 ATPase activity and co-chaperone binding. The Hsp90 chaperone cycle is in part regulated by phosphorylation mediated by casein kinase 2 (CK2), a ubiquitously expressed and constitutively active protein kinase (55, 56). Phosphorylation of yeast Hsp90-T22 (human Hsp90α-T36) or Hsp90β-S365 by CK2 disrupts binding of Hsp90 with the kinase-specific co-chaperone Cdc37 (Table 1 and Fig. 3) (64, 93). Additionally, yHsp90-T22 phosphorylation compromises Hsp90 interaction with the activating co-chaperone Aha1 and subsequently results in decreased Hsp90 ATPase activity (64). Further, ATP binding is impacted by CK2 phosphorylation of the Hsp90β charged linker, which has been suggested to precede Hsp90:Cdc37 complex dissociation when ATP levels are low (84). Interestingly, CK2 phosphorylation of Cdc37-S13 promotes its association with kinases and recruitment of Hsp90 to kinase:Cdc37:Hsp90 ternary complexes for the chaperoning of kinase clients (46–48, 152). Furthermore, sequential and ordered phosphorylation of the co-chaperone folliculin-interacting protein 1 (FNIP1) on several adjacent serine residues promotes its binding to Hsp90 and facilitates kinase and nonkinase client chaperoning (153). CK2-mediated phosphorylation of both Cdc37 and FNIP1 is specifically reversed by the phosphatase co-chaperone PP5, highlighting the complex interplay of PTMs in the chaperone machinery (47, 153).

Phosphorylation of Hsp90α-T90 by protein kinase A (PKA) also alters the complement of co-chaperones bound to Hsp90 and decreases Hsp90 affinity for ATP (73). Phosphomimetic mutation of Hsp90α-T90 amplified binding to the co-chaperones Aha1, p23, PP5, and C terminus of Hsp70-interacting protein (CHIP) but diminished binding to Hsp70, Cdc37, and HOP. In contrast, phosphorylation of residues in the Hsp90α C-domain by the kinases CK2, CK1, and glycogen synthase kinase 3β (GSK3β) increased interaction between Hsp90α and HOP while decreasing interaction with CHIP (104).

Hsp90 phosphorylation also has a consequent impact on client binding and activation. Hsp90α-T90 phosphorylation abrogated binding to the client kinases Src, Akt, and PKCγ likely as a result of diminished interaction between Hsp90 and Cdc37 (73). Interestingly, phosphorylation of yHsp90-T101 (hHsp90-T115) by the dual-specificity kinase Mps1 promoted kinase client activation, whereas the nonphosphorylatable alanine mutation favored nonkinase clients, possibly due to altered co-chaperone interactions (78). Furthermore, phosphorylation of two residues in the charged linker, Hsp90α-S231 and -S263, was reduced in cells expressing a truncated form of the co-chaperone p23 (85). Phosphorylation at these residues was important for telomerase activity through stability of the hTERT telomerase catalytic subunit, which is an Hsp90 client (85).

Phosphatases also play a critical role in modulating Hsp90 phosphorylation status and therefore the chaperone cycle. The co-chaperone Ppt1 (yeast ortholog of PP5) was shown to directly dephosphorylate Hsp90 and consequently affected its chaperone activity (154). Another study found 10 residues of yHsp90 that are dephosphorylated by Ppt1 (94). The phosphorylation status of these residues differentially influenced ATPase activity as well as client activity. Interestingly, Hsp90-S485E phosphomimetic mutant had the most robustly decreased ATPase activity despite being distant from the ATP-binding pocket, suggesting that phosphorylation of this residue may cause distant structural changes (94).

Taken together, phosphorylation is a dynamic regulatory mechanism on the Hsp90 chaperone cycle. Of note, when these sites are modified there is generally a resultant decrease in Hsp90 ATPase activity. Phosphorylation can additionally allosterically affect co-chaperone and client dynamics at binding sites far from the modified site, suggesting a complex interplay of communication across the chaperone protein. Furthermore, these allosteric effects on co-chaperone dynamics often have further consequences on Hsp90 ATPase activity, and the details of these regulatory mechanisms are not yet fully understood.

Cell cycle control—Hsp90 interacts with numerous cell cycle regulators, including the kinases CDK2, -4, and -6; Mps1 and Swe1 (yeast ortholog of human Wee1); and cyclin B (78, 106, 155–157). Hsp90α-T90 phosphorylation was found to be more abundant in actively proliferating cells (73). Phosphorylation specifically by PKA on Hsp90-T90 also promoted prostate cancer cell proliferation (74). Additionally, Hsp90α-T725 and -S726 and Hsp90β-S718 phosphomimetic mutations decreased the doubling time of HEK293 cells (104). Furthermore, Hsp90 threonine phosphorylation levels fluctuate throughout the cell cycle (78). Yeast Hsp90-T101 (hHsp90α-T115) was phosphorylated throughout mitosis, but Hsp90-T101 phosphorylation was absent in G_1 phase. The cell cycle mediators Mps1 and Cdc14 phosphorylate and dephosphorylate Hsp90-T101, respectively. Together, Mps1 and Cdc14 regulate mitotic arrest and exit from mitosis, at least in part, via Hsp90 phosphorylation (78).

DNA repair and apoptosis—DNA-dependent protein kinase (DNA-PK) is both a client and a regulator of Hsp90α that phosphorylates Hsp90α on Thr-5 and -7 (57, 58, 63). Phosphorylation of these residues occurred early in apoptosis and was critical for histone γH2AX formation at dsDNA breaks, DNA fragmentation, and apoptotic body formation (57). Furthermore, Thr-7 phosphorylation in the cytosol was a prerequisite for Hsp90α accumulation at DNA double-strand breaks and subsequent formation of DNA repair foci. Hsp90α-T7 phosphorylation level was also found to correlate with the apoptotic marker pH2AX in tumors, suggesting that Hsp90α-T7 phosphorylation could serve as a potential marker for DNA damage (63). It is unclear precisely how the role of Hsp90-T7 phosphorylation is integrated across the spectrum of DNA damage recognition and repair to apoptosis or whether these roles are context-dependent. It is noteworthy that nuclear yHsp90 was recently discovered to be important for chromatin remodeling through regulation of the Arp2/3-dependent actin filaments (158), which were found to be clustered at double-strand DNA breaks (159, 160). Interestingly, levels of pThr-5/7 increase with age and correlate with a decline in AMPK activity (58).

Table 1

Hsp90 post-translational modifications, identified modifying enzymes, and functional consequences

Identified modification sites in Hsp90 are shown. Conservation between α and β isoforms is highlighted. Further indication of other species in which residues were found as well as other modifications that particular sites are subject to is as follows: boldface type, identified in that isoform; *, identified in Hsp82; †, identified in zebrafish Hsp90a1; §, identified in *Candida albicans*; a, acetylation; n, nitration; p, phosphorylation; s, SUMOylation; sc, succinylation; u, ubiquitination; g, glycation; o, *O*-GlcNAcylation; m, methylation; c, citrullination; sn, *S*-nitrosylation; t, thiocarbamlyation; ↑, binding increased (increased ATPase or increased inhibitor binding or sensitivity); ↓, binding decreased (decreased ATPase or decreased inhibitor binding or sensitivity); nc, no change; NA, not applicable/not conserved; no entry provided in the table if not determined or examined.

PTM	Residue		Enzyme	Small-molecule binding		References
	Hsp90α	Hsp90β	Compound	ATP	Inhibitors	
Serine/threonine phosphorylation	**T5**	NA	ATM, DNA-PK			57–62
	T7	NA	ATM, DNA-PK			57–63
	T36 (*,†)	T31	CK2	↓	↑	64–66
	S63	**S58**	CK2			67–69
	T65	**T60**	CK2			68, 70
	S68	S63	CK2			68
	S72	S67	CK2			68
	T88	**T83**	PKA			69, 71, 72
	T90	**T85**	PKA	↓		72–76
	S113	**S108** (*)	HopBF1	↓		77
	T115	T110	Mps1, Cdc14, PKCγ	↓	↑	78, 79
	S164	**S159**	Cdc7-Dbf4			80, 81
	S211	**S206**	PKA/PKG			82, 83
	S231	**S226**	CK2, PP5			55, 84–89
	S263	**S255**	CK2, B-Raf, PP5			55, 84–92
	N373	**S365**	CK2			83, 93
	S399 (*)	**S391**		↓	↑	67, 94–96
	T425	S417	PKCγ	↓		79
	S460 (o)	**S452** (o)	PKA			82
	S505 (*)	**S497**		↓	↑	69, 94
	S595	**S587**	Mitogen-activated protein kinase 12 (p38γ)			97–99
	T603	**T595**	PKCγ	nc		79, 99
	S623 (*)	**S615**		↓		94, 100, 101
	T624	**T616**				102, 103
	M625 (*)	M617		↓		94
	T725	A717	CK2, CK1, GSK3β			104, 105
	S726	**S718**	CK2, CK1, GSK3β			104, 105
Tyrosine phosphorylation	**Y38** (*,n)	**Y33** (n)	Swe1	↓	↓	99, 106
	Y197	**Y192**	v-Src, Yes			107–112
	Y309	**Y301**	c-Src			110, 113, 114
	Y313	**Y305**		↑		42, 69, 107, 115
	Y627 (*)	**Y619**				69, 107, 116, 117
Acetylation	**K41** (§,u)	**K36** (u)			↑	118–120
	K69 (u)	**K64** (u)	HAT p300	↓	↑	101, 121–124
	K74 (u,g)	**K69** (u)				97, 99
	K100 (u,g)	K95	HAT p300	↓	↑	121
	K292 (u)	**K284** (u)	HAT p300		↑	121, 124
	K294 (†,§,u)	**K286** (u)	HDAC6		↑	65, 118, 119, 125–129
	K327 (u)	**K319** (u)	HAT p300	↓	↑	99, 121
	K407 (u)	**K399** (u)			↑	124, 130
	K419 (u)	K411			↑	130, 131
	K478 (u,g)	S470	HAT p300	↓	↑	121
	K546 (u,g,sc)	**K538** (u,sc)	HAT p300	↓	↑	121, 131
	K558 (u)	K550	HAT p300	↓	↑	121
Monomethylation	**K209** (†,u)	K204	SMYD2			65, 132
	K539	**K531** (a,u)	SMYD2			133
	K582	**K574** (a,u)	SMYD2			133
	K615 (*,†,a,u)	K607	SMYD2			65, 132, 134–136
Thiocarbamylation	C529	**C521**	6-HITC-ME			137
	C597 (sn)	**C589** (sn)	STCA			138
S-Nitrosylation	**C597** (t)	**C589** (t)	Nitric oxide			139, 140
SUMOylation	**K191** (a,u)	K186	SUMO-1		↑	141
	K559 (u)	K551	SUMO peptidase sentrin/SUMO-specific protease 2 (SENP2)			142
Ubiquitination	**K112** (g)	**K107**	CHIP			143–145
	K209 (m)	**K204** (a)	CHIP			143, 144, 146
	K224 (a)	**K219** (a)	CHIP			143, 144, 146
	K283 (a,g)	**K275** (a)	CHIP			143, 144, 146
	K292 (a)	**K284** (a)	CHIP			143, 144, 146
	R355	**K347** (a,m,sc)	CHIP			144
	K407 (a)	**K399** (a)	CHIP			143, 144, 146
	K485 (a)	**K477** (a)	CHIP			143, 144
	K489 (a,m,sc)	**K481** (a,sc)	CHIP			143, 144, 146
	K546 (a,g,sc)	**K538** (a,sc)	CHIP			143–145
	K558 (a)	**K550**	CHIP			143, 144
	K615 (a,m)	**K607** (a)	CHIP			143, 144, 147
	K631 (a,sc)	**K623** (a,sc)	CHIP			143, 144, 148
Nitration	**Y38** (p)	**Y33** (p)				149, 150

Table 1—*Continued*

PTM	Residue		Enzyme	Small-molecule binding		References
	Hsp90α	Hsp90β	Compound	ATP	Inhibitors	
	Y61 (p)	**Y56** (p)				149
O-GlcNAcylation	S442 (p)	**S434** (p)				151
	S460 (p)	**S452** (p)				151

There is evidence of a role for Hsp90β in apoptosis as well. Hypophosphorylation of Hsp90β-S226 and -S255 was found to prevent cytochrome *c*–induced apoptosome activation (86).

Transcription factor regulation—Numerous transcription factors have been found to interact with Hsp90, including vertebrate steroid hormone receptors and PAS domain family members. It has been shown that Hsp90 phosphorylation increased glucocorticoid receptor (GR) activity (64, 93). Mutation of yeast Hsp90-T22 to phosphomimetic glutamate increased GR activity over 4-fold compared with WT Hsp90, whereas mutation to the phospho-null Hsp90-T22A significantly decreased GR activity (64). Interestingly, phosphomimetic mutation of Hsp90β-S365E, a residue that is not conserved in Hsp90α, also significantly increased GR activity when expressed as the sole Hsp90 in yeast (93). Additionally, Hsp90-T90 phosphorylation by PKA is important for androgen receptor (AR)-mediated transcription (74). Phosphorylation of Hsp90-T90 released AR from Hsp90, leading to AR nuclear localization and subsequent transcription (74). Work by Ogiso *et al.* (87) demonstrated phosphorylation of Hsp90 residues in the charged linker (Hsp90α-S230 and Hsp90β-S226 and -S255) that impaired Hsp90 binding to the bHLH/PAS family transcription factor arylhydrocarbon receptor. Nonphosphorylatable mutation of these residues restored Hsp90 interaction with arylhydrocarbon receptor, subsequently increasing its transcriptional activity toward xenobiotic-responsive elements (87). These works highlight the importance of Hsp90 PTMs for regulating chaperoning of transcription factors and demonstrate the need for further investigation into this area.

Cancer development and progression—Consistent with cancer cells' reliance on Hsp90 chaperone machinery, Hsp90 is differentially modified in cancer *versus* normal cells, and Hsp90 phosphorylation has been linked to numerous cancer-driving processes. In addition to some examples seen above, early work in the field posited a role for Hsp90 phosphorylation in cellular transformation. Phosphatase inhibitor treatment led to hyperphosphorylation of Hsp90 and decreased complex formation between Hsp90 and the oncogenic tyrosine kinase client, v-Src, and suggested that cycling of Hsp90 PTMs could direct v-Src trafficking and cellular transformation (54). Hsp90 phosphorylation has been implicated to contribute to many of the hallmarks of cancer, including processes such as cancer cell migration (79), proliferation (74, 104), invasion (75), tumor regression and immunogenicity (90), and therapeutic resistance (86). Furthermore Hsp90α-S164 is hyperphosphorylated in oral cancer (80), and total phosphorylated Hsp90 was found to be increased in breast tumor samples compared with normal tissue (104). In contrast, Hsp90β-S226 and -S255 are hypophosphorylated in leukemic cells, further demonstrating the regulatory importance of Hsp90β PTMs (86). The mechanisms that contribute to these differential degrees of Hsp90 phosphorylation in specific instances are largely unknown. It is likely that this reflects a combination of alterations in kinase or phosphatase expression as well as changes to kinase regulation. Of note, many of the kinases that modify Hsp90 are themselves clients. Mps1, one of the client kinases, was increased in kidney cancer tissue relative to adjacent normal tissue, and this correlated with Hsp90 inhibitor sensitivity, suggesting that Hsp90 phosphorylation plays a role in kidney cancer as well (78). The effect of Hsp90 PTMs on drug sensitivity will be further discussed below.

Tyrosine phosphorylation

Chaperone cycle and kinase maturation—Numerous cellular pathways are also regulated by Hsp90 tyrosine phosphorylation. Significant work has been done detailing a series of tyrosine phosphorylation events that affect the Hsp90 chaperone cycle and chaperoning of kinase clients. Nonreceptor tyrosine kinase Yes plays a prominent role in Hsp90 function through phosphorylation of both Hsp90α and co-chaperone Cdc37. Yes first phosphorylates Cdc37-Y298, inducing a conformational change that primes the client:Cdc37:Yes complex to bind to Hsp90α (107). Next, Yes phosphorylates Hsp90-Y197, disrupting the Hsp90:Cdc37 complex (107, 108). This process is essential to kinase client maturation (108). Perhaps this is due to the subsequent phosphorylation of Hsp90-Y313 on only one protomer of an Hsp90 dimer (*i.e.* asymmetric phosphorylation), which recruits Aha1, thereby stimulating Hsp90 ATP hydrolysis and client maturation (42, 107). Interestingly, whereas phosphomimetic mutation Hsp90-Y313E enhances Aha1 association with Hsp90, it abrogates interaction with the competing co-chaperone tuberous sclerosis complex protein 1 (Tsc1) (161). Phosphorylation of Hsp90-Y627 by Yes then triggers dissociation of Aha1 and the mature client, "resetting" Hsp90 for the next cycle (107). Additionally, Hsp90-Y627 phosphorylation has been proposed as a mechanism to functionally replace the evolutionarily lost yeast Aha1-like co-chaperone, Hch1 (88). This series of Hsp90 tyrosine phosphorylation events profoundly affects the dynamics of the chaperone cycle, including the binding and release of co-chaperone proteins. Determination of precisely how this translates to client activation, however, requires further work. One example that has been elucidated is in B-cell antigen receptor (BCR) signaling. Signals transduced through BCR signaling play a role in a subset of B-cell lymphomas. Spleen tyrosine kinase (SYK), a key effector of BCR signaling, is an Hsp90 client and depends on Hsp90α-Y197 phosphorylation for its function (109). The Hsp90 client Swe1 also mediates Hsp90 tyrosine phosphorylation that affects Hsp90:co-chaperone dynamics. Phosphorylation of

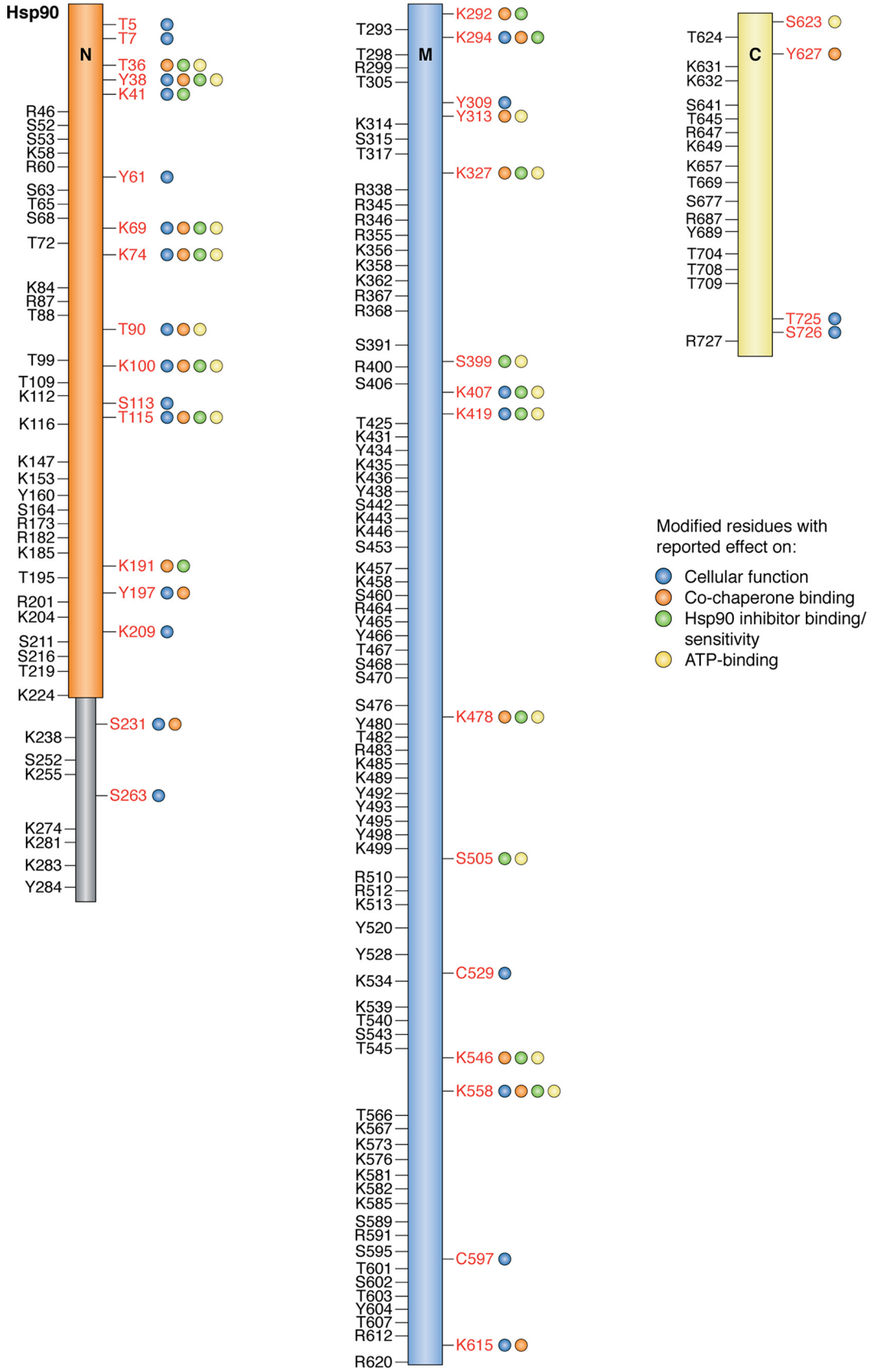

Figure 3. Schematic representation of Hsp90α PTM sites. Residues that have been functionally studied and found to contribute to the chaperone code are shown in *red* with *colored circles* indicating effects on cellular function (*blue*), co-chaperone binding (*orange*), ATP binding (*yellow*), and Hsp90 inhibitor binding/sensitivity (*green*). Residues in *black* have been reported to be modified but have not yet been validated to affect chaperone function.

yHsp90-Y24 (human Hsp90α-Y38) was important for Hsp90 interaction with the co-chaperones Aha1 and p23; knockout of Swe1 or mutation of Tyr-24 to the nonphosphorylatable phenylalanine abolished Hsp90 interaction with Aha1 and decreased interaction with p23 (106).

Cell cycle control—Phosphorylation of the conserved yHsp90-Y24 (human Hsp90α-Y38) has diverse implications on not only Hsp90 chaperone function but also the cell cycle. Swe1 (yeast ortholog of human Wee1) is a key component of the G_2-M cell cycle checkpoint (162). Swe1 phosphorylated yHsp90-Y24 in a cell cycle–dependent manner, and Hsp90 tyrosine phosphorylation was observed only in S phase–arrested cells (106). It was also shown that yHsp90-Y24 phosphorylation serves as a prerequisite to Hsp90 ubiquitination and proteasomal degradation (106). Interestingly, yHsp90-Y24 phosphorylation is also important for chaperoning of Swe1, which is an Hsp90 client. Swe1-mediated phosphorylation enhanced the association of the kinase with Hsp90 and its stability and function (163). Expression of the nonphosphorylatable yHsp90-Y24F mutant caused yeast to undergo premature nuclear division and made them insensitive to G_2/M checkpoint arrest (163).

Angiogenesis and inflammation—Additionally, Hsp90 tyrosine phosphorylation regulates cell proliferation, angiogenesis, and inflammation through endothelial nitric oxide synthase (eNOS). eNOS produces nitric oxide (NO), which mediates angiogenic and vasodilatory effects, and eNOS signaling is affected by Hsp90 phosphorylation (113). Duval *et al.* (113) found that, upon vascular endothelial growth factor receptor 2 (VEGFR2) stimulation, VEGFR2-bound Hsp90β is phosphorylated by the kinase c-Src at Tyr-300. This phosphorylation event is critical to subsequent Hsp90 interaction with eNOS and VEGF-stimulated eNOS signaling and angiogenesis. Furthermore, c-Src–mediated phosphorylation of Hsp90α-Y309 (Hsp90β-Y301) in endothelial cells could also be induced by the inflammation-inducing bacterial wall component lipopolysaccharide, and this was needed to mediate the pro-inflammatory actions of Hsp90 in this context (164). Overall, Hsp90 phosphorylation has a spectrum of effects on chaperone function and cellular processes, but there are roles for the regulatory functions of other PTMs as well.

Acetylation–deacetylation

Hsp90 chaperone function is also affected by acetylation. Acetylation is a post-translational modification that results from the addition of acetyl groups generally to lysine residues. As this modification was first identified and described on histones in the nucleus, the classes of enzymes responsible were named histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes, however, can modify numerous other cellular proteins, including Hsp90. Treatment with HDAC inhibitors (HDACi) has been demonstrated to lead to hyperacetylation of Hsp90 in numerous reports (165, 166). In general, Hsp90 acetylation alters its ability to bind ATP and Hsp90 inhibitors and its interaction with both client and co-chaperone proteins (125, 166). This has particular consequences for the stability and function of kinases and steroid hormone receptors as well as cell migration. Hsp90 inhibitor sensitivity and fungal virulence are also impacted and will be discussed later. Additionally, there are studies examining the role of Hsp90 acetylation in mediating glucocerebrosidase activity in Gaucher disease and tau phosphorylation in Alzheimer's disease (126, 167).

Oncogenic kinase chaperoning

Kinases are one of the main classes of Hsp90 clients and are needed to support growth and survival in many cancers. Early work demonstrated that inhibition of HDAC6 led to the hyperacetylation of Hsp90 and reduced the association of Hsp90 with its kinase clients, including Bcr-Abl, Akt, and c-Raf (165). Destabilization and degradation of kinases as a result of HDACi and decreased kinase association with Hsp90 has also been demonstrated for VEGFR1, VEGFR2, erythroblastic oncogene B (ErbB1), ErbB2, Raf-1, and extracellular signal–regulated protein kinase (ERK1/2) (168, 169). Hsp90-K294 acetylation specifically has been shown to affect both kinase and nonkinase client binding (125). Acetylated lysine mimic mutations K294A and K294Q disrupted interaction of Hsp90 with its clients ErbB2 and v-Src as well as the kinase-specific co-chaperone Cdc37, whereas nonacetylatable mutation K294R either enhanced binding or had no effect (125). Another interesting study demonstrated that Hsp90 acetylation can also be increased by inhibition of the transcription factor heat shock factor 1 (HSF1) as a result of disruption of a cytosolic complex containing HSF1, p97, Hsp90, and HDAC6 (170). This further resulted in reduced association of Hsp90 and Cdc37 and consequent depletion of kinase clients, including Bruton's tyrosine kinase, c-Raf, and CDK4 (170). Hsp90 function and kinase stability were also reduced following HSF1-targeted treatment in a leukemia mouse model, suggesting possible therapeutic benefit of targeting this mechanism (170).

Steroid hormone receptor stability and activity

Steroid hormone receptors are another well-described class of Hsp90 clients that are affected by Hsp90 acetylation. Some of the initial work demonstrating regulation of Hsp90 acetylation by HDAC6 showed compromised Hsp90-dependent GR maturation in HDAC6-deficient cells (166). GR ligand binding, nuclear translocation, and transcriptional activity were impaired with HDAC6 inhibition or loss (166, 171). This stemmed from a defect of hyperacetylated Hsp90 in functional heterocomplex formation with GR and not from an intrinsic change to GR (172). As a result of HDAC6 inhibition, hyperacetylated Hsp90 also dissociated from the co-chaperone p23 (95). Hsp90 acetylation–dependent GR signaling has also been implicated in stress resilience. Selective depletion of HDAC6 in serotonergic neurons decreased GR signaling and led to reduced angiogenic effects of corticosterone and also blocked the expression of social avoidance behavior in mice exposed to chronic social defeat (173). Similarly, lower levels of Hsp90 acetylation and consequent enhanced GR translocation have been seen in the dorsal raphe nucleus after chronic social defeat stress (127). Treatment with an HDAC6i, ACY-738, increased relative association of Hsp90 with the co-chaperone FKBP52 and inhibited

hormone-induced GR translocation in cells and in mice promoted resilience to chronic social defeat stress. The effects of HDACi on stress resilience could also be replicated with a Lys-294 acetylation mimetic, suggesting these effects are mediated through modulation of acetylation on this specific lysine residue (127). In contrast to the effects of Hsp90 acetylation on GR function, specifically Hsp90-K294 acetylation, another group demonstrated that acetylation of the equivalent lysine, Lys-295, in mouse Hsp90α alters the cellular localization of the mineralocorticoid receptor (MR). Interestingly, it did not alter MR transcriptional activity and therefore suggests that Hsp90 acetylation could assist in balancing MR and GR activity when both are expressed (128).

Modulation of Hsp90 acetylation also has effects on the estrogen and androgen receptors. Hsp90 hyperacetylation as a result of HDACi decreased binding of Hsp90 to the estrogen receptor (ERα) leading to its ubiquitination and proteasomal degradation (174). This led to a subsequent decrease in ER-mediated transcription. HDACi in breast cancer cells also decreased the stability of other prosurvival Hsp90 clients, including Akt and c-Raf (174). Similarly, AR dissociated from Hsp90 and was degraded in the proteasome upon treatment with sulforaphane, a derivative of glucoraphanin that has been shown to mediate HDAC6 inhibition (175). Sulforaphane increased Hsp90 acetylation, leading to AR instability and reduced AR target gene expression in an HDAC6-specific manner. HDAC6 knockdown (KD) similarly impaired AR localization and inhibited prostate-specific antigen expression in the absence or presence of the AR ligand dihydrotestosterone (176). The effect on AR localization in this study was also mediated by acetylation on Lys-294 of Hsp90 (176), and AR has been seen to dissociate from K294A or K294Q acetyl mimics (125). Overall, Hsp90 acetylation, particularly at Lys-294, plays an important role in the chaperoning and function of steroid hormone receptors. Modulation of these dynamics may present a therapeutic target in stress resilience as well as hormone-dependent cancers, such as breast and prostate cancer.

Cytoskeletal dynamics and cell migration and invasion

Several studies have examined the role of Hsp90 acetylation in affecting cytoskeletal remodeling dynamics as well as cell migration. He *et al.* (65) examined the role of various Hsp90α PTMs on myosin thick filament organization in zebrafish skeletal muscle. Hsp90 plays a key role in myosin folding and thick filament assembly, but this role was repressed by acetylation mimic K287Q (Lys-294 in hHsp90α) mutation, whereas the nonacetylatable K287R was able to rescue thick filament defects in Hsp90α KD embryos (65).

Hsp90 acetylation also plays a role in actin cytoskeleton dynamics. An unexpected finding of HDAC6 localizing to actin-enriched membrane ruffles led to the identification of Hsp90 recruitment to membrane ruffles and macropinosomes as well (177). The acetylation-resistant K294R mutant enhanced dorsal ruffle formation in HDAC6 knockout mouse embryonic fibroblasts, whereas acetyl mimic K294Q did not. Treatment with the Hsp90 inhibitor geldanamycin also inhibited ruffle formation as well as cell migration (177). Hsp90 further plays a role in mediating HDAC1-regulated cell migration through stability of TAp73, a member of the p53 family (178). HDAC1 KD resulted in hyperacetylation of Hsp90 and subsequent proteasomal degradation of TAp73, which was required for the enhanced cell migration seen with HDAC1 KD (178). Hsp90 is also a target of SIRT2, a tumor suppressor and class III histone deacetylase. SIRT2 was found to deacetylate Hsp90, and this was a necessary prerequisite to ubiquitination and degradation of Hsp90 (179). The regulation of Hsp90 by SIRT2 deacetylase activity was required for SIRT2 inhibition of actin polymerization and cell migration (179). Additionally, Hsp90 acetylation plays a role in tumor cell invasiveness. Three acetylation mimics of Hsp90α, K69Q, K100Q, and K558Q, were found to promote the export of Hsp90 and increased the amount found extracellularly (121). These acetyl mimics further facilitated binding to the matrix metalloproteinase MMP2 and enhanced invasiveness of breast cancer cells in a Matrigel invasion assay. Treatment with pan-HDACi promoted cell invasiveness; however, treatment with an anti-acetylated-K69-Hsp90 antibody inhibited invasiveness to a greater degree than a polyclonal anti-Hsp90 antibody, suggesting that specifically targeting acetylated Hsp90 in the extracellular space could decrease tumor invasion and metastasis (121). Further potential therapeutic benefits utilizing Hsp90 acetylation will be discussed below.

Other reported PTMs

As seen above, the literature examining the effects of phosphorylation and acetylation on Hsp90 function is quite robust. Many other types of PTMs, however, have also been reported to impact Hsp90 function. Here we summarize those studies, and of note, these sections are not further subdivided by functional consequence because the reports are less numerous than those examined above.

Methylation

Protein methylation has most notably been studied in nuclear histone proteins, where the so-called "histone code" regulates gene expression (180). Despite this focus, protein methylation is also a common cytosolic modification. Catalyzed by methyltransferases, this enzymatic process frequently targets lysine and arginine side chains. SET and MYND domain–containing 2 (SMYD2) is one such lysine methyltransferase, reported to methylate Hsp90α at Lys-209 and conserved Lys-615 (Hsp90β-K607, yHsp90-K594) (132, 134). Methylation of yHsp90-K594 causes allosteric conformational changes that impact Hsp90 ATP hydrolysis, Aha1 and p23 binding, and client activity (134). Specifically, monomethylated yHsp90-K594 was generated via amber suppression and demonstrated impaired N-terminal dimerization kinetics compared with WT Hsp90 (134). Additionally, SMYD2 is also reported to interact with p23 and HOP co-chaperones, suggesting varied roles for methylation in the regulation of Hsp90 function (132). Hsp90-K615 methylation in skeletal and cardiac muscle has been shown to promote the stability of the sarcomeric protein titin and myofibril organization, providing a mechanistic explanation for the role of Hsp90 in skeletal and cardiac muscle function (135, 181). SMYD2 also methylated Hsp90β at the

conserved residues Lys-531 and -574. These methylation events promoted Hsp90 chaperone function and served to increase the growth of bladder cancer cell lines (133). In summary, lysine methylation serves to enhance Hsp90 function through a wide array of modification sites and can act as a switch point to allosterically regulate distant regions of Hsp90.

Thiocarbamylation and S-nitrosylation

Thiocarbamylation of Hsp90 results from binding of the small molecules sulfoxylthiocarbamate alkyne (STCA) and 6-methylsulfinylhexyl isothiocyanate-β-mercaptoethanol (6-HITC-ME) to Hsp90 after the exogenous addition of these compounds. Treatment with these small molecules has been reported to inhibit Hsp90 function by modification of the middle domain residues Cys-597 in Hsp90α (Cys-589 in Hsp90β; STCA) (138) as well as Cys-521 in Hsp90β (6-HITC-ME) (137). Both small molecules were found to inhibit Hsp90 activity and induce degradation of client proteins. This was accompanied by the induction and nuclear translocation of the prosurvival transcription factor HSF-1, potentially blunting the cytotoxic effects of these compounds (137, 138).

Similarly to thiocarbamylation, *S*-nitrosylation results from chemical modification of cysteine residues. NO induces reversible *S*-nitrosylation by forming stable thionitrite groups on cysteine residues (139). NO is produced by the Hsp90 client eNOS in cells and is an important signal transduction molecule (182). Hsp90α-C597 was subject to nitrosylation as a result of eNOS activity, which decreased Hsp90 chaperone activity, providing a reciprocal mechanism for the regulation of eNOS in cells. It has been posited that this contributes to the anti-cancer effects of nitric oxide (139, 140).

SUMOylation and citrullination

SUMOylation is the addition of a small ubiquitin-like modifier (SUMO) protein to lysine residues. Asymmetric SUMOylation of Hsp90α-K191 by SUMO-1 recruited Aha1 to the chaperone complex and sensitized Hsp90 to its inhibitors (141). SUMOylated Hsp90 was enriched in cells that had undergone malignant transformation, providing one potential explanation for the preferential accumulation of Hsp90 inhibitors in cancer cells (141). *In vitro* cross-linking of SUMO to an introduced cysteine at the target site in yeast Hsp90 (yHsp90-K178) demonstrated an ATPase activity similar to that of the unmodified Hsp90 (183). Chemically SUMOylated Hsp90 displayed enhanced interaction with Aha1. Additionally, Sba1 (yeast p23 ortholog) was less able to compete Aha1 off and slow down Hsp90 ATPase activity (183). Interestingly, SUMOylated Hsp90α-K559 was shown to serve as an immunogen and biomarker in monoclonal gammopathies of undetermined significance, multiple myelomas, and Waldenstrom's macroglobulinemias (142). This SUMOylation state is the result of a heritable defect in sentrin/SUMO-specific protease 2 (SENP2) that renders it unable to deSUMOylate Hsp90 (142). This specific modification event may contribute to the relative success of Hsp90 inhibition in multiple myelomas (184–187).

Citrullination of Hsp90 has also been implicated in immunogenicity. Examination of sera from rheumatoid arthritis–associated interstitial lung disease patients identified citrullinated Hsp90α/β as autoantigens (188). Citrullination results from the deamination of arginine into citrulline. Numerous residues in Hsp90β have been reported as citrullinated, including the "minimum citrullination set" of Arg-392, -604, -612, and -679 (189). In the context of Hsp90, citrullination induced local unfolding, exposing cryptic epitopes that contributed to its immunogenicity (189). This almost certainly results in decreased chaperone function, although no specific impact on Hsp90 has been reported.

Ubiquitination

One of the many quality control mechanisms in cells involves the ubiquitination and proteasomal degradation of proteins. Indeed, post-translational cross-talk between phosphorylation and ubiquitination has been previously shown to induce Hsp90 turnover (66, 106).

HECT domain E3 ubiquitin protein ligase (Hectd1) participates in the ubiquitination and degradation of Hsp90 (190). Functionally, Hectd1 mutation increased Hsp90α secretion and Hsp90-dependent migration of cranial mesenchyme cells (190). Indeed, ubiquitination of Hsp90 was recently identified at numerous lysine residues using a novel detection technique (143). Interestingly, the Hsp90 co-chaperone CHIP is also an E3 ubiquitin ligase. CHIP was shown to ubiquitinate Hsp90β on 13 lysine residues, leading to Hsp90β degradation (Table 1) (144). It is noteworthy that phosphorylation of Hsp90α-T725 and -S726 and Hsp90β-S718 diminished interaction with CHIP, further demonstrating the cross-talk between Hsp90 phosphorylation and ubiquitination (104). These data point to Hsp90 ubiquitination as a critical cellular regulatory control module.

Oxidation

Tubocapsenolide A (TA) is a bioactive compound isolated from *Tubocapsicum anomalum* that demonstrates toxicity against a variety of cancer cell lines. TA induces degradation of Hsp90 client proteins, suggesting an Hsp90-inhibitory function. Mechanistically, TA treatment perturbed redox homeostasis, promoting reactive oxygen species generation and oxidation-induced aggregation of the chaperone proteins Hsp90 and Hsp70 (191). Similarly, 4-hydroxy-2-nonenal was shown to inhibit Hsp90 activity by forming thiol-adducts with Hsp90-C572 (192), further confirming redox homeostasis as a crucial factor of proteome maintenance.

Nitration

Hsp90 activity can also be inhibited by peroxynitrite, through the process of nitration (149). Nitration of two conserved N-domain tyrosine residues (Hsp90α-Y38/Y61; Hsp90β-Y33/Y56) induced apoptosis in neuronal models and was also found in spinal cord sections of ALS patients (149). These residues are in close proximity to the N-domain ATP-binding pocket of Hsp90, providing a potential mechanism for the inhibition of Hsp90 activity. Interestingly, Tyr-33–nitrated Hsp90β has also been shown to preferentially associate with mitochondria,

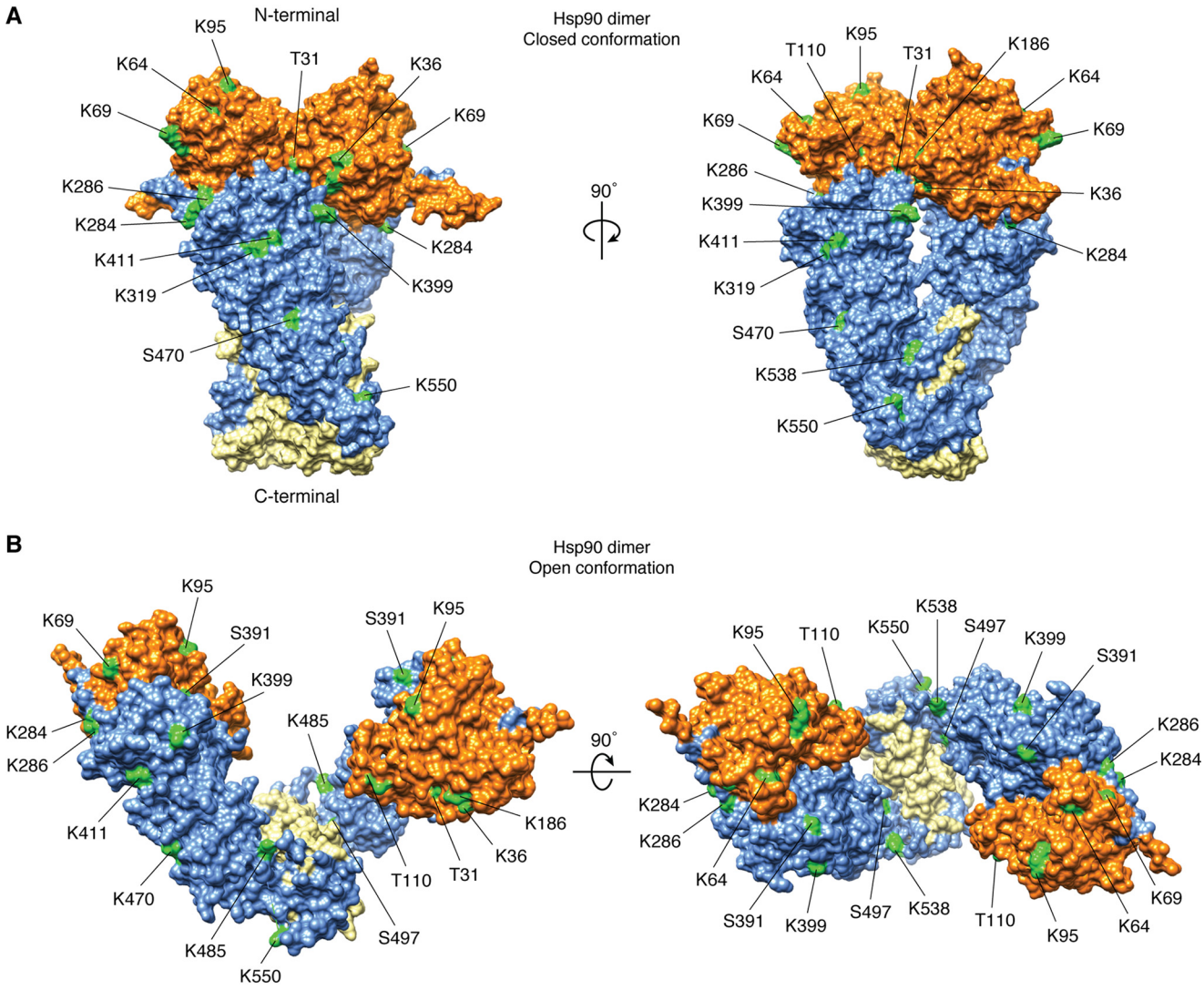

Figure 4. PTM sites known to alter Hsp90 inhibitor sensitivity. Shown is the Hsp90 dimer structure in the closed (*A*; Protein Data Bank entry 5FWP) and open (*B*; Protein Data Bank entry 2IOQ) conformation with PTM sites known to alter sensitivity to Hsp90 inhibitors highlighted (*green*), demonstrating that modifications throughout Hsp90 can profoundly influence the efficacy of N-domain inhibition. Of note, some of these residues are subject to various PTMs, only one of which has been shown to impact drug binding as detailed in Table 1.

exerting a detrimental effect on mitochondrial metabolism (150). Taken together, the down-regulation of mitochondrial function provides an explanation for the association between nitrated Hsp90 and neurodegenerative disease.

Glycosylation

O-GlcNAcylation of Hsp90 has been reported at several sites in both Hsp90α and β using a novel biotin-cystamine approach (151). Although no functional assays were performed, *O*-GlcNAc antagonizes phosphorylation of serine residues (88), suggesting specific redundant regulatory modules for the identified modification sites. *O*-GlcNAcylation of Hsp90 was later found to preferentially occur under hyperglycemic conditions. This modification decreased the ability of Hsp90 to support developmental competence in mouse oocytes in an *in vitro* maturation assay. Interestingly, this phenotype was reversed by 17-AAG treatment, suggesting a detrimental function for *O*-GlcNAcylated Hsp90, although the precise mechanism remains unknown (193). Notably, *O*-GlcNAc of the Hsp90 co-chaperone FNIP1 was recently reported, demonstrating multiple layers of Hsp90 regulation by one type of modification (153).

Other forms of glycosylation are distinct from *O*-GlcNAcylation in that they are typically irreversible. Nonenzymatic glycosylation, or glycation, involves the modification of lysine and arginine side chains with reactive sugar moieties such as methylglyoxal (Fig. 3). Several Hsp90 residues spanning all domains are reported to be glycated, inducing degradation of the Hsp90 client LATS1 and functionally inhibiting Hsp90 activity, while supporting the tumorigenesis of breast cancer cell lines (194).

Hsp90 post-translational modification and drug sensitivity

Sensitivity to Hsp90 inhibitors

As mentioned above, Hsp90 is an attractive therapeutic target, particularly in cancer, due to the reliance of key processes such as cell proliferation and survival as well as cell migration on Hsp90

chaperone function. Inhibiting Hsp90 therefore provides a unique way to simultaneously target divergent signaling pathways, including those mutated or up-regulated in cancers, such as oncogenic kinase pathways (195). Multiple studies have demonstrated increased sensitivity of cancer cells to Hsp90 inhibition relative to normal cells (196). It has also been shown that Hsp90 inhibitors preferentially accumulate in tumor tissue compared with normal tissue; however, the mechanism for this selectivity is not yet fully understood (197–199). Post-translational modification of Hsp90 is one modulatory mechanism that has been explored for Hsp90 inhibitor sensitivity and selectivity (Fig. 4, *A* and *B*).

As mentioned earlier, SUMOylation of Hsp90-K191 increased binding to and enhanced cell sensitivity to Hsp90 inhibitors (141). Additionally, cells expressing transforming oncogenic kinases exhibited an increase in Hsp90 SUMOylation and concomitant increase in Hsp90 inhibitor sensitivity.

Hsp90 phosphorylation status is often altered in cancer and has also been shown to affect sensitivity to Hsp90 inhibitors, as seen by growth inhibition or arrest or induction of apoptosis (66, 78, 80, 86, 104, 106). Phosphorylation of yHsp90-T22 increased sensitivity to various Hsp90 inhibitors, particularly at higher concentrations (66). Effects of phosphorylation on Hsp90 inhibitor binding can also vary depending on the inhibitor in question and the Hsp90 isoform. Beebe *et al.* (110) showed that PU-H71 bound Hsp90 phosphorylated on various tyrosine residues better than geldanamycin (GA). Furthermore, whereas phosphomimetic mutations on conserved Hsp90α-Y197E and Hsp90β-Y192E both disrupted binding to GA, only Hsp90β-Y301E and not Hsp90α-Y309E decreased binding to GA (110). Our group also demonstrated that Mps1-mediated phosphorylation of Hsp90α-T115 conferred sensitivity to Hsp90 inhibitors SNX-2112 and ganetespib. Additionally, Mps1 levels were elevated in renal cell carcinoma tissue, and ganetespib selectively accumulated in renal cell carcinoma tumor tissue with elevated levels of Mps1 as compared with adjacent normal renal tissue (78). Phosphorylation of Hsp90α-Y38 (yHsp90-Y24), on the other hand, decreased sensitivity to Hsp90 inhibitors, and nonphosphorylatable yHsp90-Y24F bound more inhibitor (106). Knockdown or inhibition of Wee1, the kinase responsible for Tyr-38 phosphorylation, sensitized cancer cells to Hsp90 inhibition. Dual pharmacologic treatment caused induction of the intrinsic apoptotic pathway and led to down-regulation of an inhibitor of apoptosis, Survivin, as well as Wee1 (200). Taken together, residue-specific Hsp90 phosphorylation may serve as a biomarker for the efficacy of Hsp90 inhibitors, and combination therapies may be of benefit in some cases. It is noteworthy that Tyr-38 phosphorylation is the only PTM reported to decrease Hsp90 inhibitor sensitivity (Table 1).

The potential role for combination treatments is further highlighted when examining how Hsp90 acetylation impacts Hsp90 binding to its inhibitors and inhibitor sensitivity. Overall, acetylation of Hsp90 appears to largely decrease the ATP-binding and chaperone function of Hsp90 but increases Hsp90 binding to its inhibitors (121, 201). Acetylation mimic mutations at several specific residues (Lys-69, -100, -292, -327, -478, -546, and -558) have also been shown to enhance binding of Hsp90 to biotinylated geldanamycin (121). Furthermore, co-treatment of leukemic cells with Hsp90 inhibitor 17-AAG and an HDAC6i led to greater cell death than treatment with either agent alone (201). Additionally, leukemic cells resistant to pan-HDAC inhibitors have been shown to have increased expression of HDAC1, -2, and -4, with loss of HDAC6 expression but hyperacetylation of Hsp90 (122). These cells retain sensitivity to Hsp90 inhibition (122). Recent work by our group has also demonstrated that Hsp90 acetylation affects bladder cancer cell sensitivity to Hsp90 inhibitors (130). Loss of the tumor suppressor and co-chaperone Tsc1 was associated with decreased binding to and uptake of Hsp90 inhibitors and with a decrease of Hsp90 acetylation but not phosphorylation (130, 161). Hsp90 was found to be acetylated on Lys-407 and -419, and HDACi treatment rescued Hsp90 acetylation in Tsc1 knockout cells. Ultimately, Tsc1-null bladder cancer cells were more sensitive to HDACi, and treatment with HDACi resensitized the cells and synergized with Hsp90 inhibitor treatment (130). Overall, there is a growing body of evidence that acetylation of Hsp90 diminishes its chaperone function and increases sensitivity to Hsp90 inhibitors. As a result, there may be a therapeutic advantage to combination treatment with HDAC and Hsp90 inhibitors.

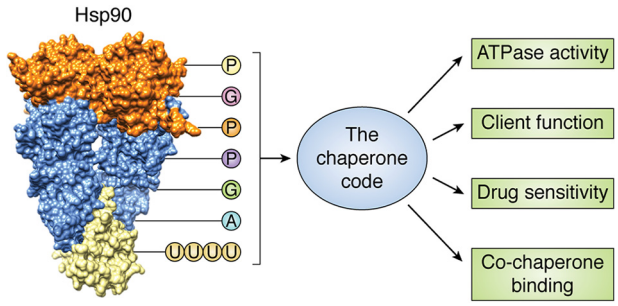

Figure 5. The Hsp90 chaperone code. Collectively, the landscape of Hsp90 PTMs, or the "chaperone code," affects Hsp90 chaperone function as defined by Hsp90 ATPase activity, co-chaperone binding, drug sensitivity, and client function.

As can be seen in the above examples, reported PTMs in general tend to increase Hsp90 inhibitor sensitivity (Table 1). This must, however, be taken into context. Whereas some modifications, such as Hsp90-T115 phosphorylation, are elevated in cancer and confer sensitivity, elevation of others like Hsp90-Y38 confers resistance. Similarly, whereas Hsp90 acetylation may serve as a marker for sensitivity, its absence provides the opportunity for co-treatment with an HDACi to restore sensitivity in an otherwise resistant cell. Collectively, these results paint a complex landscape that requires further study for mechanistic insight.

Resistance to other therapeutics

In addition to affecting sensitivity to Hsp90 inhibitors, Hsp90 modifications have also been demonstrated to affect sensitivity to other drugs and therapies. In one example, rifampin-induced CK2-mediated phosphorylation of Hsp90β-S226/255 in the charged linker led to the induction of P-glycoprotein, a drug-exporting transporter involved in multidrug resistance (89). In contrast, hypophosphorylation of the same residues conferred resistance to imatinib (Gleevec) in leukemic cells (86). This may be due to inhibition of cytochrome *c*–induced apoptosome formation through Hsp90-mediated Apaf-1 sequestration, but

the exact mechanism of imatinib resistance is unknown (86). Reduced phosphorylation at the conserved Hsp90α-S231 and -S263 also correlated with a down-regulation of telomerase activity and subsequent sensitization to cisplatin (85). These examples highlight the complexity of identifying the impact of Hsp90 PTMs, as the same sites can lead to very different consequences, depending on the context. There are also instances where Hsp90 phosphorylation is exploitable. ATM-mediated phosphorylation of Hsp90α-T5 and -T7 sensitized cells to ionizing radiation (59). Furthermore, point mutation to phosphomimetic Hsp90-S370E and -S640E increased susceptibility of cells to UV irradiation (94).

Finally, Hsp90 acetylation has also been identified in fungal species and was found to play a role in fungal virulence and drug resistance (202). In fungi, particularly in pathogenic species, fungal Hsp90 mediates resistance to anti-fungals, such as azoles, by enabling resistance mutations and stabilizing resistance-mediating protein products (203, 204). Lysine deacetylase (KDAC) inhibition blocked the emergence of Hsp90-dependent resistance to azoles in *Candida albicans* and *Saccharomyces cerevisiae* (118, 205). KDAC inhibition compromised the stability and interaction with clients, including calcineurin, which has been shown to be important for drug resistance (205). Knockdown and inhibition of KDACs also increased sensitivity to Hsp90 inhibitors or phenocopied Hsp90 inhibition in modulating resistance to azoles (118, 205). Hsp90 was found to be acetylated on Lys-27 and -270 in baker's yeast (Lys-30 and -271 in *C. albicans*), and there was functional redundancy between the KDACs Hda1, Rpd3, Hos2, and Rpd31 (118, 205). Acetylation status at Lys-27 has also been shown to be important for azole and echinocandin resistance in *Aspergillus fumigatus* (119). Acetyl mimic mutations at Lys-27 increased susceptibility to antifungals, and nonacetylatable K27R attenuated these effects (119). Ultimately, targeting Hsp90 acetylation may represent a target for antifungal resistance and understanding the role of Hsp90 acetylation may assist in the development of new antifungal therapeutics. In summary, there is strong evidence that a complete understanding of the effects of Hsp90 PTMs may allow us to better utilize a variety of therapeutics in addition to Hsp90 inhibitors.

Concluding remarks and future perspectives

Post-translational modification often impacts intracellular localization of Hsp90 and its ability to bind clients, nucleotide, co-chaperones, and also inhibitors. Collectively, these PTMs are critical for regulating Hsp90 chaperone activity and, ultimately, cellular functions mediated by affected client proteins. It has been demonstrated that these cellular effects are far-reaching and impact processes including but not limited to cell cycle control, DNA repair, cell signaling, and cell migration. Despite the vast number of reports available with regard to Hsp90 PTMs, there are still several gaps in our knowledge.

First, the vast majority of PTMs have only been explored in the context of cytosolic Hsp90. Translating these findings to organelle- and compartment-specific Hsp90 regulation will promote a more complete understanding of chaperone-mediated signaling pathways. Further, the growing appreciation for extracellular Hsp90 necessitates further research into the mechanisms regulating its secretion activity and how PTMs affect Hsp90 function extracellularly. Additionally, it is well-established that whereas Hsp90 functions as a dimer, there is a great deal of asymmetry between the protomers, and this is essential for its function. This is highlighted by one Aha1 molecule interacting asymmetrically with a dimer and being influenced by asymmetric modifications, such as Hsp90-K191 SUMOylation and Hsp90-Y313 phosphorylation (42, 141, 161). We therefore need to consider whether both protomers are modified concurrently and how modification conservation between the α and β isoforms affects chaperone function. Furthermore, outside of some reports of cross-talk between Hsp90 phosphorylation and ubiquitination, there is a void in understanding the relationships between different modifications and how they influence one another and work in concert or opposition. Last, methods for detecting Hsp90 PTMs have recently been reviewed by our group (206), but more specific methods, such as the development of phosphospecific antibodies, are warranted and likely necessary to fully unravel the chaperone code.

Ultimately, however, we need to use several orthogonal approaches to fully decipher the chaperone code (Fig. 5). Knowledge obtained from the tools used in this field to date leaves us with information on how a limited number of modifications affect consequences in specific settings. We are also lacking robust mechanistic detail of how PTMs lead to the observed changes in client chaperoning. Looking forward, we must gain an appreciation for the many different populations of Hsp90 with respect to their modification states and how they function together or independently to ultimately influence the protein landscape within cells. Understanding the cross-talk between different modifications is paramount to unraveling the regulation of chaperone function. Implementation of methods such as artificial intelligence, bioinformatics, and molecular modeling allows data processing on a much larger scale. Synthesis of a detailed, mechanistic understanding of Hsp90 modifications with population-wide analysis will allow us to fully decipher the chaperone code and translate it to therapeutic benefit.

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Abbreviations—The abbreviations used are: PTM, post-translational modification; TPR, tetratricopeptide repeat; HOP, Hsp70-

Hsp90–organizing protein; PKA, protein kinase A; CHIP, C terminus of Hsp70-interacting protein; DNA-PK, DNA-dependent protein kinase; GR, glucocorticoid receptor; AR, androgen receptor; BCR, B-cell antigen receptor; eNOS, endothelial nitric oxide synthase; HAT, histone acetyltransferase; HDAC, histone deacetylase; HDACi, HDAC inhibitor; MR, mineralocorticoid receptor; ER, estrogen receptor; STCA, sulfoxylthiocarbamate alkyne; 6-HITC-ME, 6-methylsulfinylhexyl isothiocyanate-β-mercaptoethanol; SUMO, small ubiquitin-like modifier; TA, tubocapsenolide A; 17-AAG, 17-allylamino-17-demethoxygeldanamycin; GA, geldanamycin; KDAC, lysine deacetylase; KD, knockdown.

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