Review

Paul LaPointe*, Rebecca Mercier and Annemarie Wolmarans

Aha-type co-chaperones: the alpha or the omega of the Hsp90 ATPase cycle?

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Abstract: Heat shock protein 90 (Hsp90) is a dimeric molecular chaperone that plays an essential role in cellular homeostasis. It functions in the context of a structurally dynamic ATP-dependent cycle to promote conformational changes in its clientele to aid stability, maturation, and activation. The client activation cycle is tightly regulated by a cohort of co-chaperone proteins that display specific binding preferences for certain conformations of Hsp90, guiding Hsp90 through its functional ATPase cycle. Aha-type co-chaperones are well-known to robustly stimulate the ATPase activity of Hsp90 but other roles in regulating the functional cycle are being revealed. In this review, we summarize the work done on the Aha-type co-chaperones since the 1990s and highlight recent discoveries with respect to the complexity of Hsp90 cycle regulation.

Keywords: Aha1; Aha-type; ATPase; ATPase stimulation; co-chaperones; Hsp90.

Introduction: overview of chaperones and protein folding

Proteins are involved in all cellular processes and must fold into appropriate three-dimensional conformations to carry out their functions. In many cases, proteins must transition between two or more conformations in order to carry out a task. Molecular chaperones play a fundamental role in protein folding by preventing inappropriate inter- and intramolecular interactions that can impair proper folding (Voisine et al., 2010). Most chaperones interact with elements that all proteins possess with little structural specificity. The general scheme for this is reversible interaction with hydrophobic amino acid side chains (Koldewey et al., 2017). Iterative rounds of binding and release prevent aggregation with neighboring proteins and allow for intramolecular folding to occur (Hartl et al., 2011).

Heat shock protein 90 (Hsp90)

The Hsp90 interacts with hundreds of substrate proteins called clients (Taipale et al., 2010, 2012; Eckl and Richter, 2013). In addition to its role in the nascent folding of client proteins, Hsp90 plays a regulatory role by chaperoning conformational changes necessary for activation or assembly (Xu and Lindquist, 1993, Loo et al., 1998; Xu et al., 1999). While hundreds of clients have been identified, Hsp90 clients are highly specific. Genome-wide interaction studies have shown that the majority of Hsp90 clients are E3 ligases, kinases and transcription factors (Taipale et al., 2012). Little can be inferred from the list of known Hsp90 clients regarding the basis for recognition because these clients do not appear to have any obvious primary sequence elements or structural features in common that could explain their dependence on Hsp90. Similarly, it is not obvious what physical change is promoted by Hsp90 in these clients because they do not all have the same moving parts. Whatever the basis for Hsp90 action, it is clear that Hsp90 functions in a highly regulated, ATP-dependent, client activation cycle (Obermann et al., 1998; Panaretou et al., 1998).

Hsp90 is a homodimeric protein, where each subunit of the dimer comprises an *N*-terminal ATP-binding domain, a middle domain and a *C*-terminal domain that is the primary, stable dimerization interface (Figure 1A) (Li et al., 2013; Saibil, 2013). While not folded domains *per se*, there is also a long charged linker that joins the *N* and middle domains as well as a *C*-terminal MEEVD motif (Figure 1A) (Brinker et al., 2002; Hainzl et al., 2009). Each of these elements is important for Hsp90 function.

***Corresponding author: Paul LaPointe,** Department of Cell Biology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton T6G 2H7, Alberta, Canada, e-mail: paul.lapointe@ualberta.ca. https://orcid.org/0000-0002-5465-1795
Rebecca Mercier: Department of Cell Biology, Faculty of Medicine and Dentistry, University of Alberta, Edmonton T6G 2H7, Alberta, Canada
Annemarie Wolmarans: Department of Biology, The King's University, Edmonton T6B 2H3, Alberta, Canada

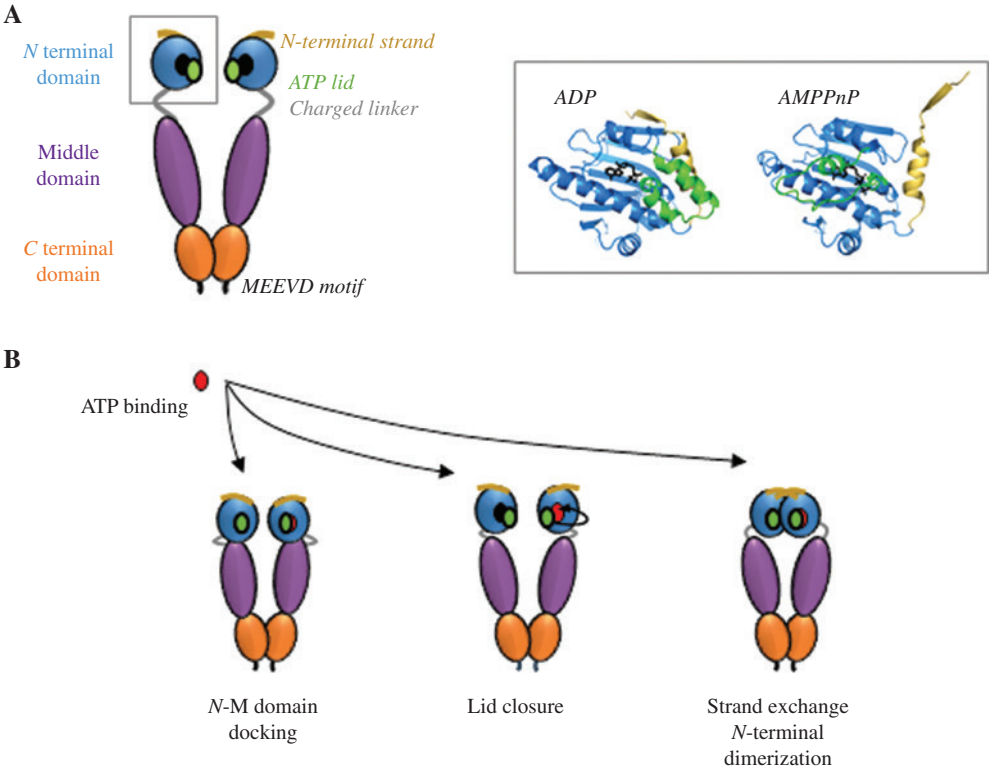

Figure 1: Structural rearrangements of Hsp90.
(A) Structure of Hsp90. Hsp90 subunits are comprised of an *N*-terminal ATPase domain (blue), connected to a middle domain (purple) by a long, charged linker, and a *C*-terminal dimerization domain (orange) which contain a MEEVD motif. Crystal structures of the *N*-terminal domain of yeast Hsp90 bound to ADP and AMPPnP nucleotides highlight two important moving elements. The ATP lid (green) has significant mobility: the *N*-terminal domain of Hsp90 bound to ADP reveals the ATP lid 'open' conformation which expose the ATP binding site, while the *N*-terminal domain bound to AMPPnP reveals the ATP lid 'closed' conformation where the lid is folded over the bound nucleotide. The *N*-terminal strands (yellow) undergo structural rearrangements, termed strand swap, during transient *N*-terminal dimerization, which is evident in the crystal structures as the strap is oriented differently when the lid is in the 'open' versus the 'closed' state. Crystal structures were modified using the PDB files 1AMW and 2CG9 for the lid open and the lid closed form. (B) ATP-induced conformational changes of Hsp90 that lead to ATP hydrolysis. ATP binding triggers three events required for ATP hydrolysis which include *N*-M domain docking, lid closure, and strand exchange.

Hsp90 acts in the in the context of a conformationally dynamic ATPase cycle (Shiau et al., 2006; Krukenberg et al., 2011; Mayer and Le Breton, 2015; Pearl, 2016). While the absolute requirement for ATP hydrolysis has recently been questioned (Zierer et al., 2016), it is clearly necessary for the efficient function of Hsp90 (Obermann et al., 1998; Panaretou et al., 1998). There are three main conformational events that occur after ATP binding that lead to hydrolysis (Figure 1B). Upon ATP binding, contact between the *N* and middle domains occur which is followed by the closure of a helix-turn-helix 'lid' motif over ATP and dimerization of the two *N* domains (Richter et al., 2001, 2006, 2008; Hessling et al., 2009; Mickler et al., 2009; Schulze et al., 2016). Binding of ATP to Hsp90 occurs when the *N*-terminal ATPase domains are in the 'open' conformation (i.e. when the *N* domain are not in contact with one another) (Prodromou et al., 2000). Contact between the *N* and middle domains after ATP binding depends on residues in the catalytic loop of the middle domain (Meyer et al., 2003). Mutation of R380 in the catalytic loop blocks lid closure and *N*-terminal dimerization, demonstrating the importance of the interaction between the middle and *N* domains (Schulze et al., 2016).

Hsp90 co-chaperones

Client activation is thought to be regulated by the sequential recruitment of proteins called co-chaperones that guide Hsp90 through numerous conformational changes (Chang et al., 1997; Fang et al., 1998; Prodromou et al., 1999; Panaretou et al., 2002; Richter et al., 2004; Siligardi et al., 2004; Armstrong et al., 2012; Lee et al., 2012; Li et al., 2012, 2013). Co-chaperones interact with Hsp90 in

different ways and play different roles in the Hsp90 functional cycle. For example, in addition to having intrinsic chaperone activity (Kimura et al., 1997), the co-chaperone cell division cycle 37 (cdc37) delivers client kinases to Hsp90 (Cutforth and Rubin, 1994; Stepanova et al., 1996; Taipale et al., 2012). There is a large group of co-chaperones that possess tetratricopeptide repeat (TPR) domains that bind to the MEEVD motif at the *C*-terminus of Hsp90 (Prodromou et al., 1999). These co-chaperones have various effects on ATP binding and hydrolysis as well as on the Hsp90 client activation cycle. For example, stress inducible 1 (Sti1) [Hsp70-Hsp90 organizing protein (HOP) in mammals] possesses three TPR domains, one of which binds to Hsp90 (Schmid et al., 2012). In both yeast and human systems, Hsp90 is held by Sti1/HOP in an open state that is competent to receive clients from the Hsp70 system (Wegele et al., 2006; Southworth and Agard, 2011; Alvira et al., 2014). Owing to the importance of *N*-terminal dimerization for ATP hydrolysis, holding Hsp90 in this open configuration also has the effect of inhibiting ATPase activity but this may be specific to yeast Hsp90 as ATPase inhibition has not been reported with human proteins (Johnson et al., 1998; Prodromou et al., 1999; McLaughlin et al., 2002; Alvira et al., 2014). Sti1 makes contact with the Hsp90 *N* domain which may block access to the nucleotide binding pocket (Prodromou et al., 1999). In contrast to Sti1, other TPR co-chaperones, such as cyclosporin-sensitive proline rotamase 6 (Cpr6) can promote nucleotide binding and hydrolysis by Hsp90 (Prodromou et al., 1999; Panaretou et al., 2002; Johnson et al., 2007). Sensitivity to benzoquinone ansamycins 1 (Sba1; p23 in mammals), is a co-chaperone that binds to the dimerized *N*-terminal domains of Hsp90 in an ATP-dependent manner (Ali et al., 2006). In single turnover ATPase reactions, Sba1 does not alter ATP hydrolysis rates but at steady state, Sba1 reduces the cycling ATPase rate (Graf et al., 2014). Presumably, Sba1 slows the rates at which hydrolyzed ATP (i.e. ADP and inorganic phosphate) is released from the ATP binding pocket because of its preference for the *N*-terminally dimerized conformation of Hsp90. The activator of Hsp90 ATPase (Aha1), which is the main focus of this review, is a co-chaperone that can dramatically accelerate ATP hydrolysis by Hsp90 which is normally very slow (Panaretou et al., 2002).

Recent work on Birt-Hogg-Dubé and tuberous sclerosis complex (TSC) syndromes has identified three new Hsp90 co-chaperones that play dual roles in regulating Hsp90 and AMPK/mTOR signaling. Tsc1 binds to the middle domain of Hsp90 where it slows ATPase activity and promotes the loading of both kinase and non-kinase Hsp90 clients (Woodford et al., 2017). Similarly, folliculin interacting proteins 1 and 2 (FNIP1 and FNIP2) bind to Hsp90, inhibit ATPase activity, and facilitate the delivery of folliculin to Hsp90 (Woodford et al., 2016a,b; Sager et al., 2019). There is a homologue of FNIP1/2 in yeast, Lst4, but whether or not it plays the same role in regulating Hsp90 is not known (Pacitto et al., 2015). The ability to facilitate the handoff of a client to Hsp90 has recently been identified in the well-characterized regulator of matrix metalloproteinase activation (Baker-Williams et al., 2019). The tissue inhibitor of metalloproteinases 2 (TIMP2) binds to the pro-form of matrix metalloproteinase 2 (MMP2) on the cell surface. TIMP2 facilitates the transfer of MMP2 to extracellular Hsp90 (eHsp90). Secreted Aha1 then displaces TIMP2 from the complex and allows for the proteolytic activation of MMP2.

The central role of Hsp90 in almost all cellular processes is consistent with the tight regulation that appears to be conferred by co-chaperones. Hsp90 is vastly more abundant than any one of these co-chaperones but likely near equal in quantity to the total co-chaperone pool (Ho et al., 2018). Thus, the function of the Hsp90 system may be very responsive to small changes in co-chaperone expression.

A brief history of Aha-type co-chaperones

Aha1 is the eponymous member for this family of co-chaperones but it was not the first to be discovered. In 1997, Hch1 (High-copy suppressor of Hsp90 mutants) was first reported in Susan Lindquist's laboratory to be able to suppress growth defects associated with a point mutant of Hsp82, namely a mutation in the catalytic loop of Hsp90, E381K (Nathan et al., 1999). Yeast that express $Hsp82^{E381K}$ as the sole source of Hsp90 grow very slowly and confers a profound defect in client activation. In that paper, *AHA1* was briefly mentioned as another gene that encoded a protein with sequence similarity to Hch1. Shortly after, Ahsa1 (i.e. mammalian Aha1) was identified in a screen for proteins that bound to the *C*-terminal, cytoplasmic tail of the model secretory protein, the vesicular stomatitis virus glycoprotein (VSVG) (Sevier and Machamer, 2001). VSVG possesses the canonical diacidic ER export motif DxE so there was great interest at the time in identifying proteins that might bind to this sequence (Nishimura and Balch, 1997). Ahsa1 was one of the few proteins that were identified in Carolyn Machamer's lab that could bind to the VSVG tail peptide and was named p38 because of its predicted molecular mass. A role for Ahsa1 in regulating

the activity or trafficking of VSVG remains to be identified. It was not until 2002 that the first paper on Aha1 was published on the role for Aha1 in stimulating the Hsp90 ATPase activity (Panaretou et al., 2002).

Conservation and curiosities of Aha-type co-chaperones

Hch1 only exists alongside Aha1 in a small group of organisms in the *Saccharomycotina* sub-phylum (Horvat et al., 2014). In most other eukaryotic organisms, only canonical Aha is present. In many cases, two paralogues can be found (called Ahsa1 and Ahsa2) which share the same canonical two-domain structure that defines the Aha co-chaperone class. There are no publications on Ahsa2 from any organism, so it is not known if it is functionally related to Ahsa1 or if Ahsa2 has a distinct function (like Hch1 in yeast).

Aha-type co-chaperones in animals possess an *N*-terminal extension that is not present in fungal and protozoan counterparts (Figure 2). This extension imparts intrinsic chaperone activity independent of co-chaperone interaction with Hsp90 (Tripathi et al., 2014). A comprehensive analysis of the evolutionary conservation of Aha co-chaperones has not been carried out, and thus, is not known how widespread this *N*-terminal extension is and whether or not intrinsic chaperone activity is conserved.

There are also reports of Aha homologues encoded in protist genomes that do not follow the canonical structure of the family. *Entamoeba histolytica* encodes an Aha co-chaperone homologue that is comprised of only one domain corresponding to the *C*-terminus of canonical Aha1 (Singh et al., 2014). It is reported to stimulate ATPase activity of Hsp90, but it is unclear how its *in vivo* function relates to Aha co-chaperones in other organisms.

Structural elements of Aha1

Aha1 comprises two domains connected by an unstructured linker of about 30 amino acids (Figure 3A) (Panaretou et al., 2002; Koulov et al., 2010). While the structure of Hch1 has not been solved, it is thought to fold into a similar structure as the Aha1 *N*-terminal domain. Aha1 interacts with the Hsp90 dimer in an antiparallel fashion where the Aha1 *N* domain interacts with the Hsp90 middle domain and the Aha1 *C* domain interacts with the Hsp90 *N* domains in an as-yet unidentified dimerized state

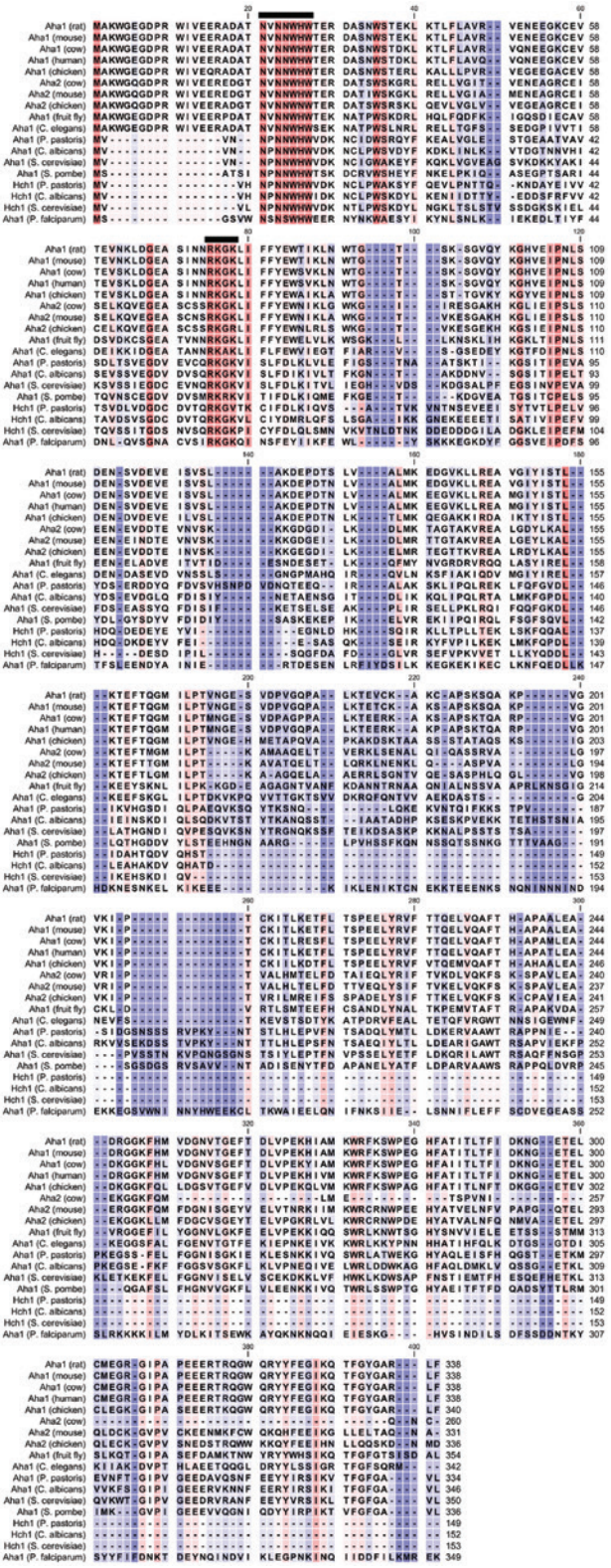

Figure 2: Sequence conservation of Aha-type co-chaperones. An alignment of the protein sequence of different members of the Aha-type co-chaperone family are shown (red/conserved – blue/divergent). The NxNNWHW and RKxK motifs are marked with black lines.

(Figure 3B) (Meyer et al., 2004; Koulov et al., 2010; Retzlaff et al., 2010). Hch1, owing to its similarity to the Aha1 *N* domain, is presumed to interact in an analogous manner with the Hsp90 middle domain (Armstrong et al., 2012). Aha1 binding to Hsp90 results in a profound increase in the ATPase rate of Hsp90 at steady state (Panaretou et al., 2002). Both Hch1 and the isolated Aha1 *N* domain stimulate the Hsp90 ATPase rate but to a far lower degree, highlighting the critical role for the Aha1 *C*-terminal domain in ATPase stimulation (Panaretou et al., 2002; Lotz et al., 2003; Meyer et al., 2004; Armstrong et al., 2012; Horvat et al., 2014; Wolmarans et al., 2016). The Aha1 *C* domain is also an important contributor to binding affinity as Hch1 and the Aha1 *N* domain bind to Hsp90 more weakly than full-length Aha1 (~5–10 fold less strongly than full-length Aha1 which in most experiments has a K_d of ~0.3 μM) (Panaretou et al., 2002; Retzlaff et al., 2010; Li et al., 2013). The mechanism for ATPase stimulation by Aha-type co-chaperones is thought to be highly conserved among eukaryotes and proceeds through a series of discrete steps (Richter et al., 2008; Vaughan et al., 2009).

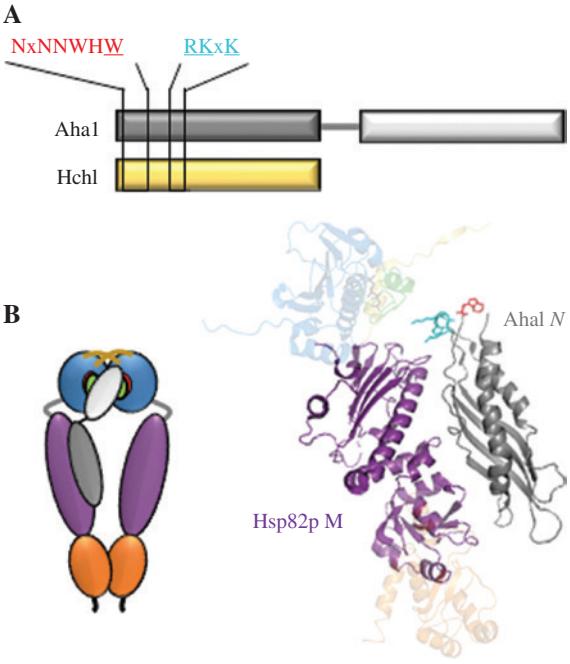

Figure 3: Structure of Aha-type co-chaperones.
(A) Domain structure of yeast Aha1 and Hch1. Aha1 (gray) is a two-domain protein, while its homolog, Hch1 (yellow), only consists of one-domain which shares 36.6% amino acid identity to the *N*-terminal domain of Aha1. The NxNNWHW (red) and RKxK (cyan) motifs are the only sequence motifs found in all known members of the Aha-type co-chaperone family. The underlined residues in each motif are shown in the crystal structure. (B) Aha1 binding to Hsp90. Aha1 binds in an anti-parallel fashion to Hsp90; Aha1 *N* domain (dark gray) binds Hsp90 middle domain (purple) and the Aha1 *C* domain (light gray) binds Hsp90 *N*-terminal domains (blue). The ribbon crystal structure of Aha1 *N* domain bound to the middle domain of Hsp90 highlight the upward orientation of the conserved motifs in (A). The crystal figure was constructed using PDB file 2CG9 that show the *N*-terminal (blue) and *C*-terminal (orange) domain of Hsp90 in alignment with PDB file 1USV of the middle domain of Hsp90 bound to Aha1 *N* domain.

There are two strongly conserved motifs in Aha-type co-chaperones (Figure 3A). The RKxK motif resides in a loop that, when Aha1 is bound to Hsp90, is near the Hsp90 *N*-terminal domains and the basic side chains are oriented towards the catalytic loop in the Hsp90 middle domain (residues 375-388) (Figure 3B) (Meyer et al., 2003). While the mechanism is not completely understood, stabilizing the catalytic loop promotes the interaction of the ATP-bound Hsp90 *N* domains with the Hsp90 middle domain (where the catalytic loop resides). Mutation of R380 abolishes events that occur downstream of ATP binding (lid closure, *N*-terminal dimerization), suggesting that interaction of this amino acid with the gamma phosphate of ATP occurs first and is a requirement for these downstream events (Schulze et al., 2016). Once the *N* domains associate with the middle domains via the catalytic loop-ATP interaction, the lid closes over bound ATP. This event is thought to expose hydrophobic residues in the *N*-terminal domains that interact to stabilize the dimerized and catalytically active state (Prodromou, 2016). ATPase stimulation by Aha1 is driven by dramatically accelerating *N*-M communication, lid closure, and *N*-terminal dimerization in Hsp90 (Figure 1B) (Hessling et al., 2009; Schulze et al., 2016). Despite its low intrinsic ATPase rate, once the catalytically competent state of Hsp90 has been acquired, it is in fact a 'perfect' enzyme (Lee et al., 2019). It remains to be determined precisely how the individual residues of the RKxK motif participate in the acquisition of the catalytically competent state. Even less clear is how acceleration of each step in the conformational cycle contributes to client activation by Hsp90 in cells. Numerous studies report the poor correlation between ATPase rates and *in vivo* activity. Mutation of any residue in the RKxK motif in Hch1 impair *in vitro* ATPase stimulation but only mutation of K60A appears to impair Hch1 action in yeast cells (Horvat et al., 2014). Indeed, ATPase defects associated with Hsp90 point mutations do not correlate with *in vivo* activity (Hawle et al., 2006; Zierer et al., 2016).

Another conserved element in Aha-type co-chaperones, the *N*-terminal NxNNWHW motif, regulates the apparent affinity for ATP and nucleotide exchange in Hsp90 (Figure 3A) (Mercier et al., 2019). Deletion of the NxNNWHW motif in Aha1 impairs steady state ATPase activity but has no effect on the acquisition of the catalytic conformation of

Hsp90. However, loss of this sequence element eliminates *in vivo* activity of Aha1. Therefore, Aha-type co-chaperones possess the ability to regulate biologically relevant events both before *and* after catalysis (Figure 4).

Genetic interactions of Aha-type co-chaperones

Yeast is one of the most powerful model systems employed to understand the role of co-chaperones in Hsp90 biology. Early work revealed that the deletion of *HCH1* and *AHA1* conferred temperature sensitive growth to yeast (Lotz et al., 2003). Yeast expressing different Hsp90 point mutants provide more detailed information regarding the role of co-chaperones in specific conformational transitions in the ATPase or client activation cycle. These mutants display specific impairments in conformational transitions that occur in the client activation cycle. As mentioned, Hch1 was identified as a high copy suppressor of the E381K mutation of Hsp90, along with co-chaperones cyclophilin seven suppressor (Cns1) and Sti1 (Nathan et al., 1999). Overexpression of Hch1 rescues normal growth of yeast expressing Hsp82^{E381K} and deletion is synthetically lethal with this mutant (Armstrong et al., 2012; Horvat et al., 2014). Curiously, neither overexpression nor deletion of Aha1 affect yeast expressing Hsp82^{E381K}, suggesting these two co-chaperones, despite their similarities, do not regulate Hsp90 in the same way (Armstrong et al., 2012). Furthermore, the E381K mutation impairs ATPase stimulation by Aha1 but not by Hch1 *in vitro* (Horvat et al., 2014). This functional divergence is also observed with Hsp82^{G313S} and Hsp82^{A587T} (Armstrong et al., 2012). These two mutations both confer temperature sensitive growth to yeast that is modified by Hch1, but not Aha1, expression. Interestingly, a Hsp90 mutant has recently been identified that is affected by Aha1. Yeast that express Hsp82^{S25P} exhibit temperature sensitive growth that is suppressed by Aha1 overexpression (Mercier et al., 2019). The ability of Aha1 to rescue the activity of Hsp82^{S25P} is entirely dependent on the NxNNWHW motif.

In addition to point mutations, Hsp90 variants with altered linker length are specifically affected by Aha1. The linker joining the *N* and M domains in yeast, human, and *Plasmodium falciparum* Hsp90 differ in both sequence and length (short, medium, and long, respectively) (Taipale et al., 2012). Hsp82 harboring the *P. falciparum* charged linker sequence confers growth and client activation defects in yeast that are suppressed by Aha1 overexpression. Curiously, endogenously expressed Aha1 was not stably associated with yeast Hsp90 harboring the *P. falciparum* charged linker. How exactly the charged linker influences the interaction between the catalytic loop in the middle domain and the ATP binding pocket has yet to be clarified but it is clear that Aha1 plays a role in this process.

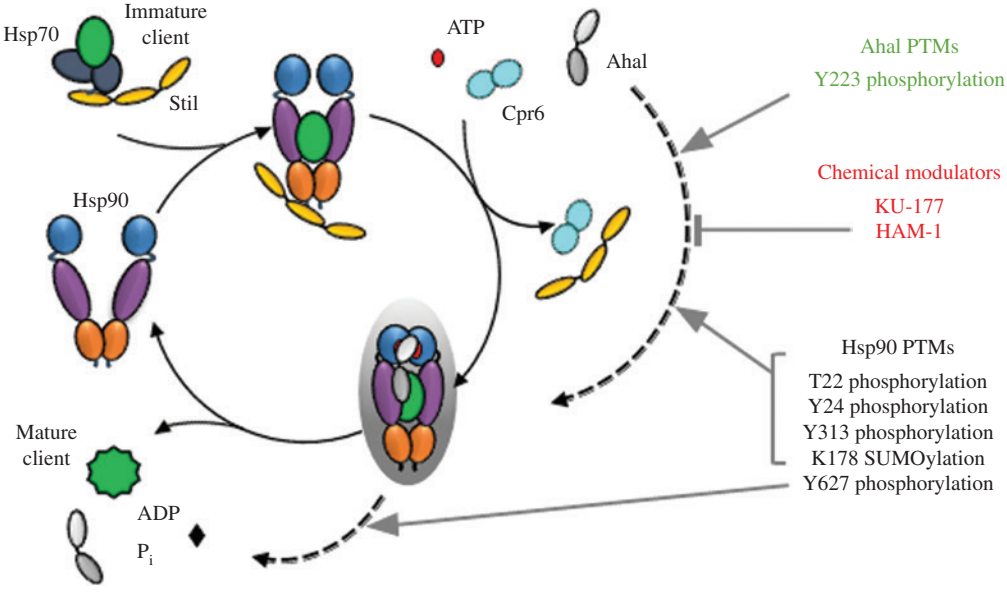

Figure 4: A simplified overview of Aha1 recruitment to the Hsp90 client activation cycle.
Aha1 plays a complex role in the functional cycle of Hsp90. Aha1 promotes the acquisition of the ATP hydrolysis competent conformation (grey oval) and participates in nucleotide release upon hydrolysis. Numerous PTMs modulate Aha1 recruitment and release, including Hsp90 (black) and Aha1 (green) PTMs. Chemical inhibitors (red) can also specifically target Aha1 from acting on the Hsp90 system, demonstrating how small chemical molecules can be used to manipulate the folding of clients for therapeutic benefit.

Asymmetric Aha1 interaction and role in co-chaperone cycling

The dimeric structure of Hsp90 means that there are two, presumably identical, co-chaperone binding sites as well as modification sites on each dimer (Flynn et al., 2015). However, potentially owing to the high abundance of Hsp90 relative to proteins that regulate it, a single binding or modification event is often sufficient to exert an effect on Hsp90 function. For example, a single molecule of Aha1 is sufficient to fully stimulate the ATPase activity of Hsp90, suggesting that Aha1 can regulate both subunits simultaneously (Retzlaff et al., 2010; Wolmarans et al., 2016). This asymmetry is likely enabled by cooperativity between the two subunits of the Hsp90 dimer (Wortmann et al., 2017). The robust stimulation of the Hsp90 ATPase activity by Aha1 suggests that it acts at or near the end of the client activation cycle. There are numerous examples of cooperative and competitive co-chaperone binding to Hsp90 with respect to Aha1. Aha1 binding is cooperative with Cpr6 (Harst et al., 2005; Li et al., 2013). This may be due to the ability of Cpr6 to enhance the affinity of Hsp90 for ATP which in turn enhances the affinity of Aha1. Aha1 can also bind to Hsp90 at the same time as Cdc37 (Gaiser et al., 2010). However, co-chaperones such as Sti1 that are known to act at the beginning of the cycle (to facilitate client transfer from Hsp70 to Hsp90) (Wegele et al., 2006), hold Hsp90 in a conformation that cannot be recognized by Aha1 (Harst et al., 2005). Aha1 competes with FNIP1 and 2, TIMP2 and Tsc1 for binding to Hsp90 (Woodford et al., 2016a,b, 2017; Baker-Williams et al., 2019; Sager et al., 2019). The Sba1 binding site on Hsp90 overlaps with much of the Aha1 binding site (Ali et al., 2006; Retzlaff et al., 2010) but there is conflicting evidence regarding their mutual exclusivity (Harst et al., 2005). It is conceivable that an Aha1-Hsp90-Sba1 heterocomplex could form where only one site is occupied by each co-chaperone (Sun et al., 2012). How Hsp90 cycles sequentially through different co-chaperone states is beginning to come into view.

Sti1 interacts through one of its three TPR domains with the *C*-terminal MEEVD motif of Hsp90 (Scheufler et al., 2000; Brinker et al., 2002). This interaction antagonizes ATP binding and holds the *N* domains apart in an 'open' conformation. The MEEVD is a docking site for other co-chaperones that possess a TPR domain (Chen and Smith, 1998; Chen et al., 1998). Owing to the dimeric nature of Hsp90, there are two MEEVD docking sites in each dimer so ternary complexes can form where Sti1 is bound to one MEEVD motif while the other is bound by a different TPR co-chaperone. Cpr6, and to a lesser extent Cpr7, can bind in such a manner (Li et al., 2011, 2013). In this conformation, Aha1 can bind to Hsp90 and displace Sti1 but not Cpr6. The mechanics of this selective displacement is not understood but it requires both the *N* and *C* domains of Aha1 (Wolmarans et al., 2016). Neither Hch1 nor the *N*-terminal domain of Aha1 on its own can displace Sti1. However, full length Aha1 as well as a chimera comprising Hch1 and the Aha1 *C*-terminal domain can displace Sti1 in cooperation with Cpr6. Consistent with its role in events that occur after nucleotide hydrolysis, the NxNNWHW motif of Aha1 is not required for cooperative displacement of Sti1 (Mercier et al., 2019).

Regulation of Hsp90 and Aha1 interaction by post-translational modifications (PTMs)

Numerous PTMs have been detected in Hsp90 and Hsp90 co-chaperones but relatively few have been studied in detail. A few PTMs of Hsp90 have been identified that modulate the recruitment of Aha1 in cells. Wee1-mediated phosphorylation of Hsp90 at Y24 in yeast (Y38 in human Hsp90α) promotes the association of Aha1 (Mollapour et al., 2010). Hsp90 that cannot be phosphorylated at Y24 (a Y24F mutant) does not bind stably to Aha1 and knockout of Wee1 also compromises the recruitment of Aha1 to Hsp90. This modification is conserved between yeast and humans and appears to have the same role in each species. Similarly, phosphorylation of T22 in yeast (T36 in human Hsp90α) by casein kinase II (CKII) promotes the interaction of Aha1 with Hsp90 (Mollapour et al., 2011a,b). Blocking CKII-mediated phosphorylation of T22 by mutation (T22A) or by disruption of CKII function destabilizes the Aha1 interaction. Curiously, the phosphosimilar substitution, Y24E, also disrupts Aha1 recruitment to Hsp90. This is also true for Mps1-mediated phosphorylation of Hsp90 at T101 in yeast (T115 in humans) where substitutions to either non-phosphorylatable or phosphosimilar residues blocks Aha1 binding (Woodford et al., 2016a,b). A simple explanation for this could be that these phosphosimilar substitutions do not properly mimic phosphorylation (which is very common). However, an intriguing possibility is that this modification only modulates Aha1 recruitment when it occurs on one subunit of the Hsp90 dimer. This is the case for SUMOylation of Hsp90 at K178 in yeast (K191 in human Hsp90α) where only one subunit of the Hsp90 dimer is SUMOylated in cells (Mollapour et al., 2014;

Wolmarans et al., 2018). Asymmetric SUMOylation is sufficient to promote Aha1 recruitment to Hsp90. This mode of regulation is consistent with the asymmetric ATPase stimulation mechanism where only one molecule of Aha1 is required for stimulation of the Hsp90 ATPase activity *in vitro* (Retzlaff et al., 2010). Similar to SUMOylation of K178, phosphorylation of Y313 on only one subunit in a dimer of human Hsp90 α is sufficient to recruit Aha1 (Xu et al., 2012, 2019). Aha1 may be subsequently displaced from Hsp90 by phosphorylation at Y627 (Xu et al., 2012).

Modification of Aha1 itself can also regulate interactions with Hsp90. Phosphorylation at position Y223 in Aha1 confers higher affinity for Hsp90 (Dunn et al., 2015). This modification allows for Aha1 to overcome the tight binding of the inhibitory factor, Tsc1, to Hsp90 and allow for ATPase stimulation in cells (Woodford et al., 2017).

Role of Aha1 in disease

The fate of Hsp90 client proteins with respect to folding, maturation, stability and activation is dictated by regulators of the ATPase cycle (Hartl et al., 2011; Saibil, 2013). Modulating certain Hsp90/co-chaperone interactions can have powerful effects on the balance between folding and degradation of Hsp90 clients (Wang et al., 2006; Mollapour et al., 2014). Aha1 accelerates the rate-limiting step(s) in the acquisition of the catalytically competent, *N*-terminally dimerized state of Hsp90 (Figure 4) (Richter et al., 2008; Koulov et al., 2010; Retzlaff et al., 2010; Li et al., 2013; Schopf et al., 2017). Any enhancement in the recruitment of Aha1 would presumably reduce the dwell-time at intermediate steps of a now-shortened cycle. Recent work has highlighted the importance of cycle timing in client processing (Zierer et al., 2016).

Aha1 can influence disease pathology through its ability to modulate the Hsp90 ATPase activity. Aha1 can promote the activation of kinases that are involved in oncogenesis (Lotz et al., 2003; Harst et al., 2005; Holmes et al., 2008). However, Aha1 activity can adversely affect client maturation, specifically for difficult-to-fold clients such as the cystic fibrosis transmembrane conductance regulator (CFTR) (Wang et al., 2006; Koulov et al., 2010). Mutations in the CFTR chloride channel cause cystic fibrosis. Deletion of a single phenylalanine at residue 508 in CFTR (ΔF508), the most common disease-associated variant, results in rapid degradation and loss of CFTR at the cell surface. Downregulation of Aha1 stabilizes the CFTR ΔF508 mutant and conversely, over-expression of Aha1 promotes the degradation of both wildtype and mutant CFTR. Similarly, impairment of PTMs that drive Aha1 recruitment to Hsp90 can also alter CFTR folding and stability (Mollapour et al., 2011a,b, 2014). This is consistent with a model where prolonged association between Hsp90 and CFTR, at intermediate stages of the cycle, can promote proper folding while premature ATP hydrolysis and cycle termination may target CFTR for degradation. Aha1 has also been implicated in disease pathology related to tau aggregation. Tau is a microtubule-associated protein that function in regulating axonal transport and stabilizing microtubules (Wang and Mandelkow, 2016; Guo et al., 2017; Shelton et al., 2017a,b). The accumulation of tau aggregates is associated with the family of neurodegenerative diseases, including Alzheimer's disease (Lee et al., 2001). Hsp90 plays a critical role in regulating the aggregation of tau both *in vitro* and *in vivo* (Dickey et al., 2007; Luo et al., 2007; Blair et al., 2013a,b). The mechanism for this appears to be dependent on the ATPase activity of Hsp90 and aggregation is promoted by ATPase enhancement by Aha1 (Shelton et al., 2017a,b). All of this supports the notion that subtle modifications of the Hsp90 cycle by PTMs or co-chaperones have profound effects on the outcome for individual client proteins. Strategies to tune Hsp90 function by targeting co-chaperones may represent a promising approach for targeting specific clients that are relevant to disease.

Chemical modulation of Aha1 action

ATP-competitive Hsp90 inhibitors have been explored as anti-cancer agents since the late 1990s but recent research has focused on targeting more specific aspects of co-chaperone regulation (Kim et al., 2009; Trepel et al., 2010; Neckers and Workman, 2012, 2014). There are at least two inhibitors of the Hsp90-Aha1 interaction. One inhibitor, KU-177, binds to the *C*-terminal, novobiocin binding site and blocks Aha1 binding to Hsp90 (Ghosh et al., 2015). Such novobiocin-based molecules are likely to allosterically affect the binding of more than one co-chaperone to Hsp90 but it is noteworthy that KU-177 inhibits tau aggregation in cells (Shelton et al., 2017a,b). A series of modulator compounds were identified in a screen centered around interdomain rearrangements in Hsp90 (Stiegler et al., 2017). One of these compounds, HAM-1, binds directly to the Aha1 *N* domain and prevents binding to the Hsp90 middle domain. HAM-1 is a potent inhibitor of Aha1-mediated conformational changes in Hsp90 as well as ATPase stimulation. In cells, HAM-1 inhibits glucocorticoid receptor activity and CFTR degradation. Another

study described the ability of inhibitors of Hsp90-Aha1 complex formation to restore chloride ion channel activity of ΔF508 CFTR (Ihrig and Obermann, 2017). Intriguingly, molecules that mimic Aha1 binding can bind to Hsp90 and accelerate ATPase activity (Zierer et al., 2014). These studies demonstrate the potential of modulators of the Hsp90 system to more precisely target specific client folding defects for therapeutic benefit.

Future outlook

Since their discovery 20 years ago, Aha-type co-chaperones have been studied almost solely as stimulators of the Hsp90 ATPase cycle (Nathan et al., 1999; Panaretou et al., 2002). As our understanding of the mechanism underlying ATPase stimulation has grown, the poor correlation between ATPase activation and client folding has become clear (Hawle et al., 2006; Armstrong et al., 2012; Zierer et al., 2016). Current work is now focused more on the balance between different physical stages of the client activation cycle and how these may differ for individual client proteins. However, Aha-type co-chaperones appear to play an additional role in nucleotide exchange (Mercier et al., 2019). There could be three distinct steps where Aha-type co-chaperones could alter the balance of the client activation cycle – nucleotide binding at the beginning of the cycle, ATP hydrolysis near the end of the cycle, and/or nucleotide release after ATP hydrolysis has taken place. Making the story even more complicated are the PTMs that occur in Hsp90 and co-chaperones that may temporally disable or delay any number of these events on a client-specific basis (Mollapour et al., 2010, 2011a,b, 2014; Xu et al., 2012, 2019; Dunn et al., 2015). Future work will undoubtably reveal how different regulatory elements are integrated and controlled to promote the folding of client proteins. This knowledge will finally yield a complete picture of how Hsp90 participates in cellular proteostasis.

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