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Hsp90 phosphorylation, Wee1, and the cell cycle

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Abstract

Heat Shock Protein 90 (Hsp90) is an essential molecular chaperone in eukaryotic cells, and it maintains the functional conformation of a subset of proteins that are typically key components of multiple regulatory and signaling networks mediating cancer cell proliferation, survival and metastasis. It is possible to selectively inhibit Hsp90 using natural products such as geldanamycin (GA) or radicicol (RD), which have served as prototypes for development of synthetic Hsp90 inhibitors. These compounds bind within the ADP/ATP-binding site of the Hsp90 N-terminal domain to inhibit its ATPase activity. As numerous N-terminal domain inhibitors are currently undergoing extensive clinical evaluation, it is important to understand the factors that may modulate in vivo susceptibility to these drugs. We recently reported that $Wee1^{Swe1}$-mediated, cell cycle-dependent, tyrosine phosphorylation of Hsp90 affects GA binding and impacts cancer cell sensitivity to Hsp90 inhibition. This phosphoryfiglation also affects Hsp90 ATPase activity and its ability to chaperone a selected group of clients, comprised primarily of protein kinases. Wee1 regulates the G_2/M transition. Here we present additional data demonstrating that tyrosine phosphorylation of Hsp90 by $Wee1^{Swe1}$ is important for $Wee1^{Swe1}$ association with Hsp90 and for $Wee1^{Swe1}$ stability. Yeast expressing non-phosphorylatable yHsp90-Y24F, like *swe1*Δ yeast, undergo premature nuclear division that is insensitive to G_2/M checkpoint arrest. These findings demonstrate the importance of Hsp90 phosphorylation for proper cell cycle regulation.

Keywords

heat shock protein 90; phosphorylation; wee1 kinase; molecular chaperones; post-translational modification; cell cycle

Introduction

Heat Shock Protein 90 (Hsp90) is one of the most abundant proteins in cells (1–2% of total cellular protein). The cellular functions of this essential molecular chaperone have been most clearly identified in mammalian cells, Drosophila and baker's yeast.[1–4] Hsp90 and a discrete set of co-chaperones create and maintain the functional conformation of a subset of proteins referred to as "clients" (www.picard.ch/downloads/Hsp90interactors.pdf).[5,6] These targets are key mediators of signal transduction and cell cycle control.

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Hsp90 function has been the subject of intense scrutiny because (1) drugs that inhibit its function are anti-tumor agents currently being evaluated in numerous clinical trials,[7] and (2) Hsp90 acts as a 'genetic buffering system' to limit the effects of genetic variation in populations. Disturbance of Hsp90 function diminishes this buffering and allows genetic variation to manifest itself, principally as morphogenic alteration.[4,8,9]

Hsp90 structure is highly conserved across species and it consists of: (1) an N-terminal domain, containing nucleotide and drug binding sites; (2) a middle (M) domain, which provides binding sites for client proteins and various co-chaperones; (3) a C-terminal domain, containing a dimerization region that provides for constitutive association of two Hsp90 protomers.[10–12] Eukaryotic Hsp90 also possess an unstructured charged-linker region of significant but variable length connecting N and M domains[13,14] (Fig. 1). Hsp90 function relies on ATP binding and hydrolysis, which in turn impact its conformational dynamics. Hsp90 inhibitors currently undergoing clinical trial halt the chaperone cycle by replacing ATP in Hsp90's nucleotide binding pocket.[12,15–18]

The regulation of Hsp90 function is complex and multifactorial.[19] In eukaryotes, co-chaperones modulate its intrinsic ATPase activity.[20,21] Post-translational modification of Hsp90 (e.g., phosphorylation, acetylation and S-nitrosylation) also impacts ATP and co-chaperone binding,[22–24] thus providing a further layer of regulation to the Hsp90 cycle not found in bacteria, a requirement no doubt made necessary by the increasingly complex utilization of Hsp90 in maintaining cellular homeostasis in the face of diverse environmental fluctuation.

Here we will briefly review serine, threonine and tyrosine phosphorylation of Hsp90, including our recent observation that Hsp90 is tyrosine phosphorylated by its client protein $Wee1^{Swe}$.[1] We will also present additional data showing that Hsp90 tyrosine phosphorylation is important for $Wee1^{Swe1}$ stability, and that yeast expressing $Wee1^{Swe1}$-non-phosphorylatable Hsp90 share a similar cell cycle defect as *SWE1* delete yeast.[25] These findings support an important role for Hsp90 in regulating the cell cycle.[25,26]

Serine/Threonine Phosphorylation of Hsp90

Hsp90 is a phosphoprotein.[27–39] However our understanding of the role played by phosphorylation of distinct residues in regulating the chaperone function of Hsp90 remains incomplete. A number of serine and threonine phosphorylation sites on Hsp90 have been identified and studied for their impact on chaperone function (Table 1).[22] Early work showed that treating cancer cells with the serine/threonine phosphatase inhibitor okadaic acid promoted Hsp90 hyperphosphorylation, which was accompanied by decreased association with its client kinase $pp60^{v\text{-}src}$, suggesting a link between Hsp90 phosphorylation and chaperoning of its client proteins.[27,35] Hsp90 is also a substrate for DNA-dependent protein kinase, Akt, B-Raf and casein kinase II (CKII).[36,37,40,41] Further, PKA phosphorylation of Thr90 induced by 3-hydroxy-3-methylglutarylcoenzyme A reductase inhibitors has been reported to increase association of human Hsp90α with the client protein eNOS.[42] Lastly, a study reported that protein phosphatase 5 (Pp5/Ppt1) can

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dephosphorylate Hsp90 in vitro.[43] This study also showed that Ppt1 deletion in yeast compromised Hsp90 activity.

Simple baker's yeast, *Saccharomyces cerevisiae*, is a well-established and valuable tool for studying various aspects of conserved protein chaperone machinery. Yeast have provided us with a powerful tool to study Hsp90 phosphorylation, since it readily allows plasmid exchange whereby any introduced Hsp90 gene—provided it is partially functional—can provide 100% of the Hsp90 of the cell. Such genetic manipulations are simply not achievable in cultured mammalian cells. Using the yeast system, it is possible to show that Hsp90 is constitutively phosphorylated on serine and theronine residues. However, Hsp90 threonine phosphorylation is lost upon either heat shock stress or treatment with the Hsp90 inhibitor geldanamycin (GA) (Fig. 2). These results agree with a previous study showing rapid dephosphorylation of Hsp90 in heat-shocked HeLa cells.[44] Loss of threonine phosphorylation may impact Hsp90 function in response to heat shock stress or to inhibitory drugs.

Tyrosine Phosphorylation of Hsp90

There are only few reports of Hsp90 tyrosine phosphorylation (Table 1).[27] A recent study reported that c-Src directly phosphorylates Tyr300 of human Hsp90β.[32,33] This is essential for VEGF-stimulated endothelial nitric oxide synthase (eNOS) association with Hsp90 and thus is necessary for nitric oxide release from endothelial cells.[33]

Another study reported that tyrosine-phosphorylated Hsp90 repressed the function of ionotropic $P2X_7$ receptors. These receptors serve as ligand-gated ion channels and are responsible for ATP-dependent lysis of macrophages.[31]

Our recent work has shown that $Wee1^{Swe1}$ phosphorylates Hsp90. Swe1 is the only "true" tyrosine kinase in budding yeast.[45] It phosphorylates and inhibits the kinase activity of the main cell cycle cyclin-dependent kinase Cdc28 (human Cdc2) thereby regulating the G_2/M transition.[46–50] Initial studies showed that in *Scizosaccharomyces pombe*, formation of an active Wee1 tyrosine kinase depends on its interaction with Hsp90.[26] Subsequent work showed that inhibiting Hsp90 chaperone function with GA led to the proteasome-mediated degradation of $Wee1^{Swe1}$.[51,52]

$Wee1^{Swe1}$ Dependent Tyrosine Phosphorylation of Hsp90 Regulates its Chaperone Function

$Wee1^{Swe1}$ phosphorylates a conserved tyrosine residue (Y24 in yHsp90 and Y38 in hHsp90α) in the N-domain of a subpopulation of nuclear-localized Hsp90 in a cell cycle-dependent manner.[25] Cytosolic relocation of phosphotyrosyl yHsp90 precedes its ubiquitination and degradation by proteasomes. This appears to be the "switching off" mechanism for this form of the chaperone, since we were unable to identify a tyrosine phosphatase capable of dephosphorylating yHsp90 (Fig. 3).

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Phosphorylation of Hsp90 by Wee1^{Swe1} is not essential for yeast survival but the non-phosphorylatable Hsp90 mutant (yHsp90-Y24F) fails to interact with the co-chaperone Aha1 and shows significantly reduced interaction with p23/Sba1 (Fig. 1). Identical results were also observed with the human Hsp90 mutant (Y38F) in mammalian cells. These data suggest that phosphorylation of Y24/Y38 affects the equilibrium between open and closed states of Hsp90. Importantly, some clients, including the protein kinases pp60$^{v\text{-}Src}$, Raf-1 (Ste11 in yeast), ErbB2, Mpk1/Slt2 (yeast MAP kinase), and the transcription factor heat shock factor 1 (yeast Hsf1), seem to require Wee1^{Swe1}mediated tyrosine phosphorylation of Hsp90. However, Hsp90 chaperoning of other clients, including the glucocorticoid receptor (GR) and androgen receptor (AR), appears not to be affected by the phosphorylation status of this residue. This is the first demonstration that phosphorylation permits Hsp90 to switch from an inactive to an active state for chaperoning of a subset of clients while not impacting the chaperoning of other client proteins.[25]

As part of this study, we found that yHsp90-Y24F, although having a similar in vitro affinity for GA (Kd 3.6 ± 0.3 μM) as wild-type yHsp90 (Kd 2.6 ± 0.3 μM), demonstrated more drug binding in vivo, as did wild-type Hsp90 expressed in *swe1*Δ yeast cells. These data are complemented by enhanced growth sensitivity of the mutants to GA compared to wild-type cells (Fig. 4). These observations in yeast were corroborated in cancer cells, where silencing of *WEE1* or pharmacologic inhibition of Wee1 kinase sensitized cells to Hsp90 inhibitor (Fig. 3).[25]

Cell Cycle Consequences of Hsp90 Tyrosine Phosphorylation

Treating yeast cells with Hsp90 inhibitors destabilizes Swe1 (Fig. 5). Interestingly, GA treatment of yeast cells for 1 hr, significantly reduces yHsp90 tyrosine phosphorylation and also increases the percentage of cells in G_1, suggesting a possible direct impact on Swe1. Indeed, we found Swe1 to be poorly expressed in yHsp90-Y24F yeast (Fig. 6A). We next overexpressed Swe1-GST, under a galactose inducible promoter (*GAL1*), in wild-type and yHsp90-Y24F yeast. Association of Swe1-GST with yHsp90-Y24F was markedly reduced compared to wild-type yeast cells (Fig. 6B). Taken together, these data suggests that Wee1^{Swe1}-dependent tyrosine phosphorylation of Hsp90 is important to strengthen the Wee1^{Swe1}-Hsp90 chaperone complex and to permit Hsp90 to effectively chaperone this tyrosine kinase.

Deletion of *SWE1* causes a short delay in entry into mitosis but the length of G_2 is unaltered. Flow cytometric analysis (FACS) showed that asynchronously growing yHsp90-Y24F mutants and *swe1*Δ cells both had a similar proportion of cells with 1C and 2C DNA content compared to wild-type cells (Fig. 7A). We then arrested these cells in G_1-phase with α-factor and then released them by incubation in fresh media containing 50 μM Latrunculin-A (Lat-A) in order to trigger checkpoint-mediated G_2 arrest. Unlike wild-type cells, the yHsp90-Y24F mutants underwent premature nuclear division, as did *swe1*Δ cells (Fig. 7B). These data suggest that yHsp90-Y24F mutants, like *swe1*Δ cells, have a defective G_2/M cell cycle checkpoint. This is fully consistent with the observed destabilization of Swe1 in yHsp90-Y24F cells. Previous reports have suggested that proteolytic destruction of Swe1 is

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the key step in its deactivation and allows entry into mitosis.[53,54] Our data implicate Hsp90 phosphorylation status (because it regulates Hsp90-Swe1 association) in this process.

Concluding Remarks

In eukaryotes, the regulation of Hsp90 function is complex. Phosphorylation events have been shown to fine tune Hsp90 chaperone activity.[2,27,33,55,56] Our recent work uncovered a unique role for $Wee1^{Swe1}$ in regulating Hsp90. We identified a single conserved tyrosine residue in the N-domain of Hsp90, whose phosphorylation status likely permits prolonged association of Hsp90 with some of its client proteins. We also demonstrated that lack of phosphorylation at this tyrosine residue enhanced Hsp90 binding to inhibitory drugs. Here, we show that, as is the case in cancer cells, prevention of this tyrosine phosphorylation makes yeast cells hypersensitive to Hsp90 inhibition. We also provide additional data suggesting that the stability of $Wee1^{Swe1}$ not only depends on its interaction with Hsp90, but also on its ability to phosphorylate this molecular chaperone. These observations demonstrate an unexpected role for $Wee1^{Swe1}$ in regulating Hsp90 function and, consequently, in determining its own ability to regulate the G_2/M checkpoint.

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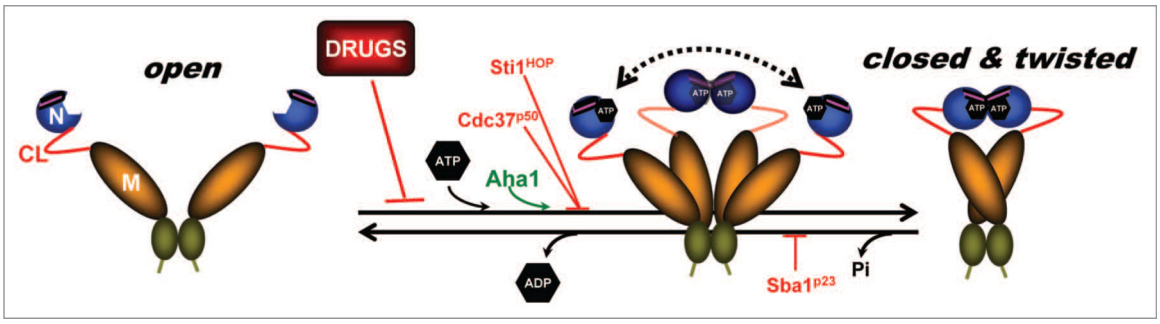

Figure 1.

Model depicting the Hsp90 chaperone cycle. ATP binding to the N-terminal domains of Hsp90 (open) promotes repositioning of a “lid” segment followed by transient dimerization of the N-domains. Subsequent structural rearrangements result in the (closed and twisted) conformation of Hsp90 that is competent for ATP hydrolysis. Binding of the co-chaperone Aha1 enhances Hsp90 ATPase activity. The co-chaperones Sti1/HOP and Cdc37/p50, or pharmacologic inhibitors such as geldanamycin or radicicol, exert an opposite effect by blocking the initial structural changes necessary for N-domain dimerization. Sba1/p23 strengthens the late Hsp90 conformation and inhibits ATP hydrolysis. Domain labeling is as follows: N, N-domain (blue); CL, charged linker (red); M, M-domain (yellow); C, C-domain (green); ATP lid, (purple).

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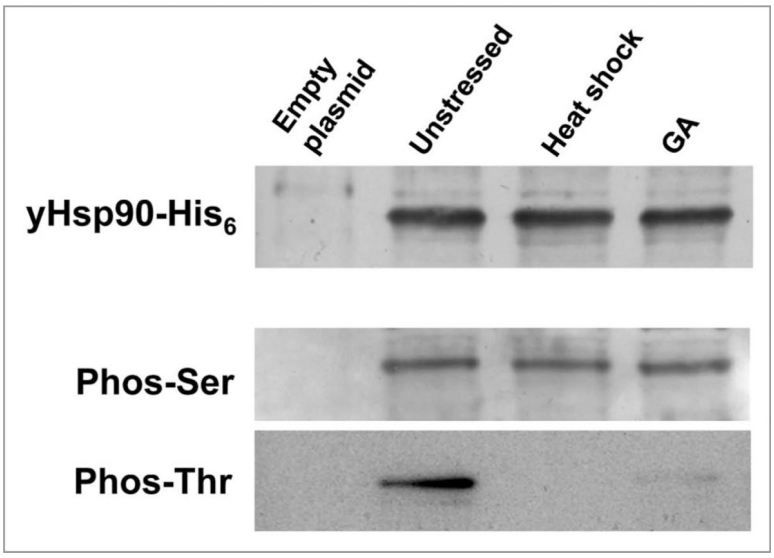

Figure 2.

Yeast Hsp90 phosphorylation on serine (phos-Ser) and threonine (phos-Thr) residues. yHsp90-His_6 was purified from yeast cells that were heat shocked at 39°C for 40 min or treated with 100 μM geldanamycin (GA) for 60 min. wild-type cells containing empty plasmid were used as negative control.

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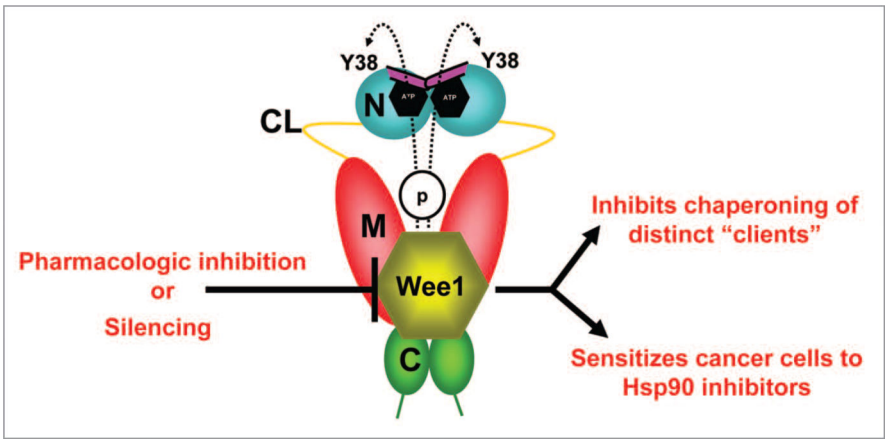

Figure 3.

wee1, an Hsp90 client protein, phosphorylates a conserved tyrosine residue (Y38) in the N-domain of a subpopulation of nuclear-localized yHsp90. Phosphorylation also leads to ubiquitination and degradation of Hsp90 by cytoplasmic proteasomes. Pharmacologic inhibition/molecular silencing of wee1 inhibits Hsp90 chaperoning of distinct clients and sensitizes cells to Hsp90 inhibitor-induced apoptosis. Domain labeling is as follows: N, N-domain (blue); CL, charged linker (yellow); M, M-domain (red); C, C-domain (green); ATP lid, (purple).

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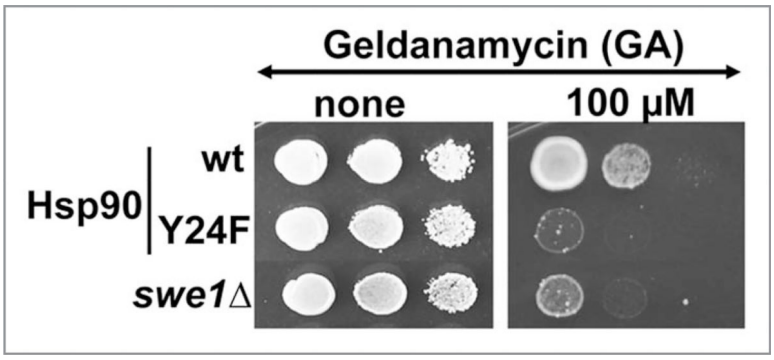

Figure 4.

Yeast cells expressing yHsp90-Y24F and *swe1*Δ cells are hypersensitive to GA. Yeast cells were grown to mid-log and then a 1:10 dilution series were spotted on YPD agar containing 100 μM GA. Plates were incubated at 25°C for 4 days.

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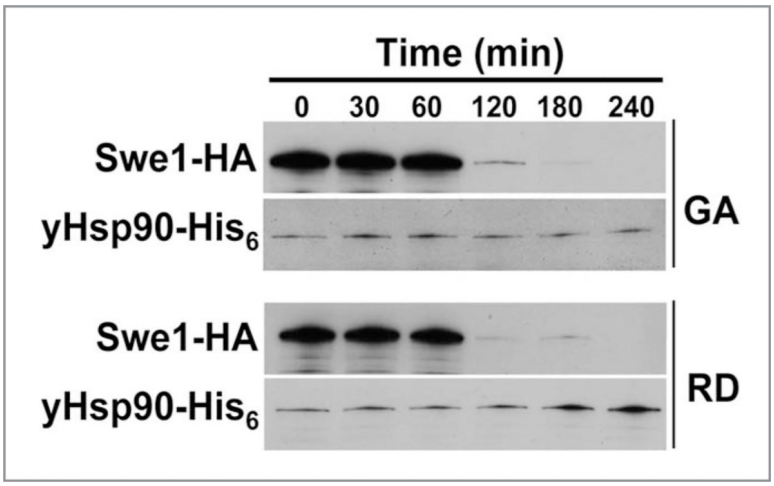

Figure 5.

Effect of Hsp90 inhibitors GA or radicicol (RD) on the stability of Swe1 tyrosine kinase. Yeast cells were grown to mid-log and then treated with 50 μM GA or RD. Swe1-HA in yeast lysate was detected by western blot using an anti-HA-monoclonal antibody. yHsp90-His_6 was used as loading control.

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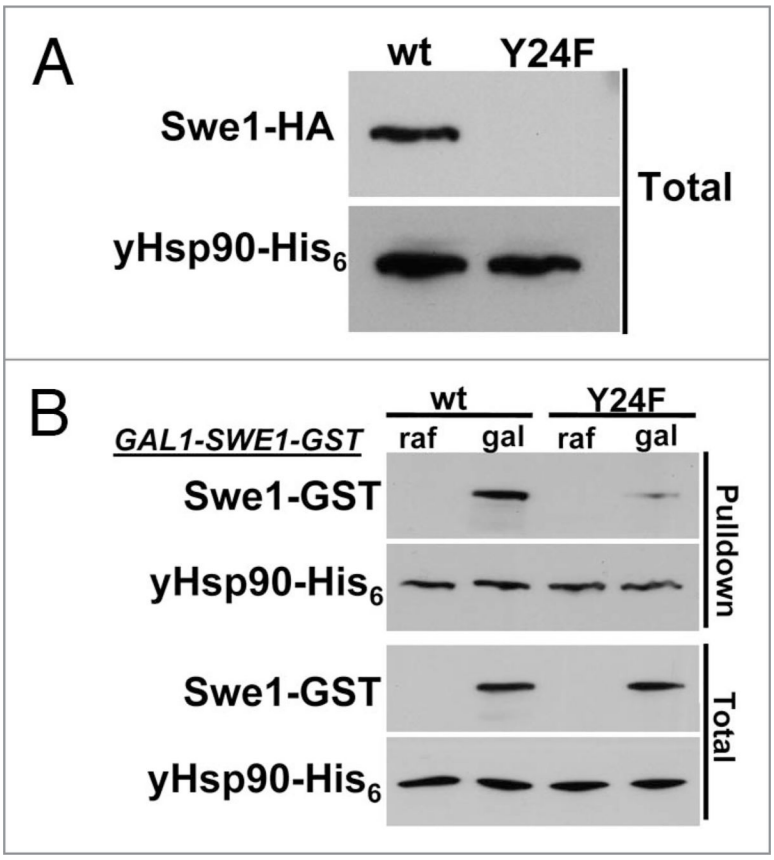

Figure 6.

Swe1 destabilization in yHsp90-Y24F-expressing yeast. (A) western blotting was used to detect Swe1-HA in yeast cell lysate expressing either wild-type yHsp90 or yHsp90-Y24F. yHsp90-His_6 was used as loading control. (B) Association of GST-tagged Swe1 with wild-type yHsp90 and yHsp90-Y24F. GST-tagged Swe1 under galactose (gal) inducible promoter (*GAL1*) was expressed in yeast cells containing either wild-type yHsp90 or yHsp90-Y24F. Swe1-GST co-precipitating with yHsp90-His_6 was detected by western blotting.

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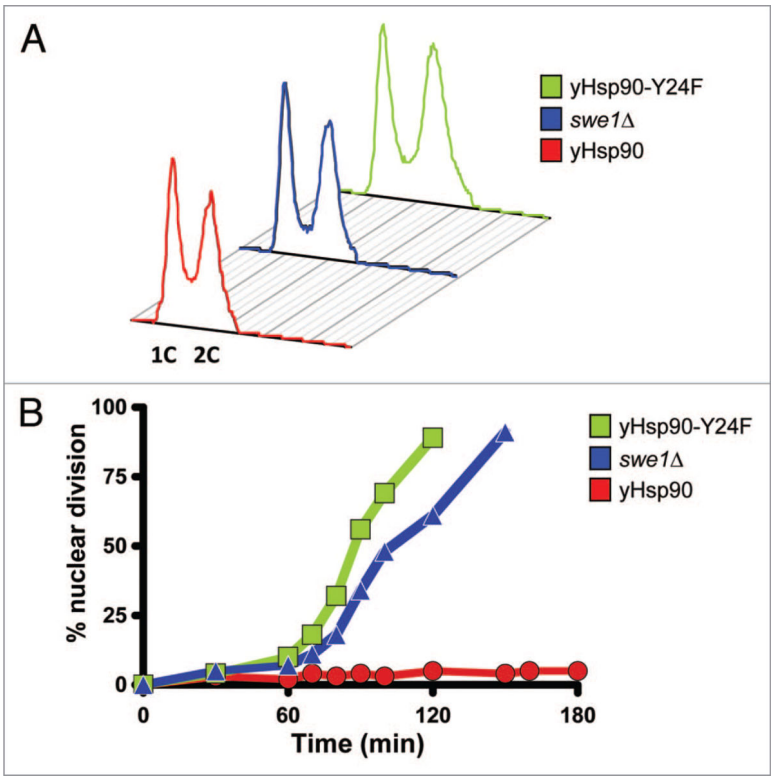

Figure 7.

Lack of G_2/M checkpoint-induced delay of nuclear division in yHsp90-Y24F and *swe1*Δ cells. (A) Flow cytometric analysis of the DNA content of asynchronously growing wild-type, *swe1*Δ, and yHsp90-Y24F yeast cells. Occupancy of G_2 is unaltered in the two mutants when compared to wild-type cells (wild-type, 48.7%; *swe1*Δ, 49.0%; yHsp90-Y24F, 51.8%). (B) Cells were released from α-factor-induced cell cycle arrest into fresh medium containing 50 μM Lat-A. inclusion of Lat-A causes arrest at the G_2/M checkpoint. At the indicated times, cell aliquots were removed, fixed and stained with DAPi to visualize DNA, and 100 cells were scored. Premature nuclear division is apparent in both yHsp90-Y24F mutant and *swe1*Δ cells.

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Table 1.

Yeast (Hsc82, Hsp82) and human (Hsp90α, Hsp90β) phosphorylation sites and identified kinases

Residues					
yHsp90	**yHsc90**	**hHsp90α**	**hHsp90β**	**Protein kinase**	**Reference**
N/A	N/A	T5*	N/A	Ds-DNA activated protein kinase	36
N/A	N/A	T7*	N/A	Ds-DNA activated protein kinase	36
Y24*	Y24	Y38*	Y33	Swe1/Wee1	25
Y47	Y47	Y61	Y56*	Unknown	57, 58
S49	N/A	S63*	S58	Casein kinase II	38
S51	S51	T65*	T60	Casein kinase II	38
N/A	N/A	S68*	S63	Casein kinase II	38
T58	T58	S72*	S67	Casein kinase II	38
N/A	N/A	T88*	T83	Unknown	42
N/A	N/A	T90*	T85	PKA	59
Y184	Y184	Y197	Y192*	Unknown	58
N/A	N/A	S231*	S226*	Casein kinase II	35, 37, 39
N/A	N/A	S252*	N/A	Unknown	60
N/A	N/A	S263*	S255*	Casein kinase II, B-Raf	35, 37, 39, 40
S282*	S278	N/A	N/A	Unknown	SGD
T285	T281	T305	T297*	Unknown	61
S297*	S293	N/A	N/A	Unknown	SGD
Y289	Y285	Y309	Y300*	c-Src (does not exist in yeast)	33
S295	S291	S315	S307*	Unknown	62
S334*	S330	N/A	N/A	Unknown	SGD
S379*	S375	S399	S391	Unknown	SGD
T443*	T429	N/A	N/A	Unknown	SGD
N/A	N/A	S460	S452*	PKA	63

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Residues					
yHsp90	**yHsc90**	**hHsp90α**	**hHsp90β**	**Protein kinase**	**Reference**
Y472	Y468	Y492*	Y484*	Unknown	PhosphoSitePlus®
Y473	Y469	Y493*	Y485	Unknown	PhosphoSitePlus®
N/A	N/A	N/A	S532*	Unknown	62
N/A	N/A	Y604*	Y596*	Unknown	PhosphoSitePlus®
Y606	Y602	Y627*	Y619*	Unknown	PhosphoSitePlus®
S616*	S612	N/A	N/A	Unknown	SGD
S619*	S615	N/A	N/A	Unknown	SGD
S657*	S653	S677	S668	Unknown	64
S663*	S659	N/A	N/A	Unknown	64
N/A	N/A	T725*	N/A	Unknown	62
N/A	N/A	S726*	S718*	Unknown	62

Identified phosphorylation residues are marked by asterisk (*); orthologue residue is also shown when present. Data were taken from published literature and the websites PhosphoSitePlus® (www.phosphosite.org) and Saccharomyces Genome Database (SGD) (www.yeastgenome.org).