Structural and functional coupling of Hsp90- and Sgt1-centred multi-protein complexes

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Sgt1 is an adaptor protein implicated in a variety of processes, including formation of the kinetochore complex in yeast, and regulation of innate immunity systems in plants and animals. Sgt1 has been found to associate with SCF E3 ubiquitin ligases, the CBF3 kinetochore complex, plant R proteins and related animal Nod-like receptors, and with the Hsp90 molecular chaperone. We have determined the crystal structure of the core Hsp90–Sgt1 complex, revealing a distinct site of interaction on the Hsp90 N-terminal domain. Using the structure, we developed mutations in Sgt1 interfacial residues, which specifically abrogate interaction with Hsp90, and disrupt Sgt1-dependent functions *in vivo*, in plants and yeast. We show that Sgt1 bridges the Hsp90 molecular chaperone system to the substrate-specific arm of SCF ubiquitin ligase complexes, suggesting a role in SCF assembly and regulation, and providing multiple complementary routes for ubiquitination of Hsp90 client proteins.

Introduction

The Hsp90 molecular chaperone, in collaboration with a plethora of co-chaperones, is involved in the assembly and stabilization of key regulatory proteins in the eukaryotic cell (Pearl and Prodromou, 2006). Although the protein clientele of Hsp90 covers a broad range of structural classes, it is nonetheless selective and specific; the biochemical basis for this remains one of the least understood aspects of Hsp90 biology. Recruitment of one important class of Hsp90 clients, protein kinases, requires the adaptor co-chaperone Cdc37, which interacts simultaneously with the kinase and Hsp90, and regulates Hsp90s ATPase cycle in the process (Roe *et al*, 2004; Pearl, 2005; Caplan *et al*, 2007). Coupling of the Hsp90 chaperone system to the ubiquitin-regulated signalling and targeted destruction systems is mediated by a dimeric co-chaperone adaptor, CHIP (Connell *et al*, 2001), which binds to Hsp90 through its tetratricopeptide repeat (TPR) domain and E2 ubiquitin conjugating enzymes through its U-box domain (Zhang *et al*, 2005). Other co-chaperones, such as p23 and Aha1, have no apparent role in mediating interactions with clients or other systems, but exert an effect instead as regulators of Hsp90s inherent ATPase cycle (Sullivan *et al*, 1997; Panaretou *et al*, 2002; Meyer *et al*, 2004; Ali *et al*, 2006).

One of the most recently recognized co-chaperones of Hsp90, Sgt1 (Takahashi *et al*, 2003), was originally identified as a suppressor of a temperature-sensitive defect in the budding yeast Skp1 protein (Kitagawa *et al*, 1999). Sgt1p associates with Skp1p as an essential component of the CBF3 kinetochore complex, and is required for the assembly of yeast and human kinetochores (Bansal *et al*, 2004; Steensgaard *et al*, 2004). Sgt1p also associates with Skp1p in the context of the SCF (Skp1p–Cdc53p–F-box protein) class of E3 ubiquitin ligases (Kitagawa *et al*, 1999). Sgt1 is not exclusively associated with Skp1 and interacts independently of Skp1 with adenylate cyclases (Dubacq *et al*, 2002; Schadick *et al*, 2002), the CHORD-domain protein RAR1 (Azevedo *et al*, 2002), and members of a family of proteins including plant disease resistance *R*-gene products (Bieri *et al*, 2004; Leister *et al*, 2005) and animal Nod-like receptors (Mayor *et al*, 2007; da Silva Correia *et al*, 2007), that mediate innate immunity to parasites and infection. The ability of Sgt1 to bind to Hsp90 and simultaneously interact with other proteins (Takahashi *et al*, 2003; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006; Mayor *et al*, 2007; da Silva Correia *et al*, 2007) implicates it as a client protein adaptor co-chaperone in a similar manner to Cdc37, albeit with a more structurally varied clientele.

Sgt1 consists of three identifiable domains: an N-terminal tetratricopeptide repeat (TPR) domain similar to those found in many other Hsp90 and Hsp70 co-chaperones; a central 'CHORD and Sgt1' (CS) domain related to the β-sandwich domain of small heat-shock proteins, α-crystallin and the Hsp90 co-chaperone p23/Sba1; and a C-terminal Sgt-specific (SGS) domain. All three domains mediate protein–protein interactions. The SGS region has been implicated in interaction with leucine-rich repeat domains in the yeast adenylate cyclase Cdc35p (Dubacq *et al*, 2002), plant R proteins (Bieri

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et al, 2004; Leister *et al*, 2005) and the mammalian Nod1 receptor (da Silva Correia *et al*, 2007). Although initial studies implicated the TPR domain as essential for interaction with both Hsp90 and Skp1 (Bansal *et al*, 2004), further analysis suggests that the N-terminal TPR domain mediates interactions with Skp1, whereas the central CS domain is responsible for the recruitment of Sgt1 to Hsp90 (Lee *et al*, 2004; Lingelbach and Kaplan, 2004; Catlett and Kaplan, 2006). Immunoprecipitation and yeast two-hybrid (Y2H) assays identify the N-terminal half of Hsp90 as sufficient for interaction with Sgt1 (Takahashi *et al*, 2003).

To gain some insight into the coupling of the Hsp90 and Sgt1 protein interaction 'hubs', we have determined the crystal structure of the Hsp90-N–Sgt1-CS core complex. The structure reveals a hitherto unknown locus of interaction on Hsp90, and facilitates a targeted mutational analysis that reveals the Hsp90 dependence of Sgt1 functions *in vivo*. Biochemical analysis of Sgt1 interactions suggests a role for Hsp90–Sgt1 in the assembly of SCF ubiquitin ligase complexes.

Results

We explored a range of Hsp90 and Sgt1 constructs from different species and obtained diffracting crystals of the core Hsp90–Sgt1 complex using an N-terminal domain construct of *Hordeum vulgare* (barley) Hsp90 and a CS domain construct from the *Arabidopsis thaliana* homologue, AtSgt1a. HvHsp90 is 64% identical to yeast Hsp82 and 70% identical to human Hsp90β, whereas AtSgt1a is 32 and 36% identical, respectively, to its yeast and human homologues. The structure of the complex was solved by molecular replacement with the crystal structure of yeast Hsp90-N domain and the NMR structure of human Sgt1-CS domain. The crystals con-

Table I Crystallographic statistics

Data collection	HvHsp90-N–AtSgt1a-CS
Space group	P2$_1$2$_1$2$_1$
a, b, c (Å)	100.268, 129.654, 135.998
Wavelength (Å)	0.9537
Resolution (Å)	38.1–3.3 (3.48–3.3)
R_{merge}	0.161 (0.561)
$I/\sigma I$	8.5 (2.3)
Completeness (%)	99.5 (99.9)
Redundancy	3.6 (3.6)
Refinement	
Number of reflections	50 605
R_{work}/R_{free}	0.20/0.24
Number of atoms	
Protein + ADP	7404
B-factors	
Protein + ADP	59.7
r.m.s.d.	
Bond lengths (Å)	0.012
Bond angles (deg)	1.483
Ramachandran statistics	
Preferred	88.8%
Allowed	8.7%
Outliers	2.4%

tain three independent copies of the complex, each with a bound ADP molecule, and has been refined at 3.3-Å resolution (see Materials and methods and Table I).

The structure of yeast and human Hsp90-N domains has been described earlier (Prodromou *et al*, 1997; Stebbins *et al*, 1997) and the barley Hsp90-N domain is essentially identical in structure (r.m.s.d. ~0.75 and 0.67 Å, respectively for 198 and 172 common Cα positions). The mobile 'lid' segment, which closes over the nucleotide-binding pocket in the ATP-bound closed state of the Hsp90 (Ali *et al*, 2006), is in a fully open conformation in the Hsp90-N–Sgt1-CS complex, and an ADP molecule is present in the nucleotide-binding pocket. The AtSgt1a CS domain has the same β3–β4 'sandwich' structure seen in the NMR structure of HsSgt1-CS domain (Lee *et al*, 2004), with small differences in the conformation of the loops connecting the β-strands.
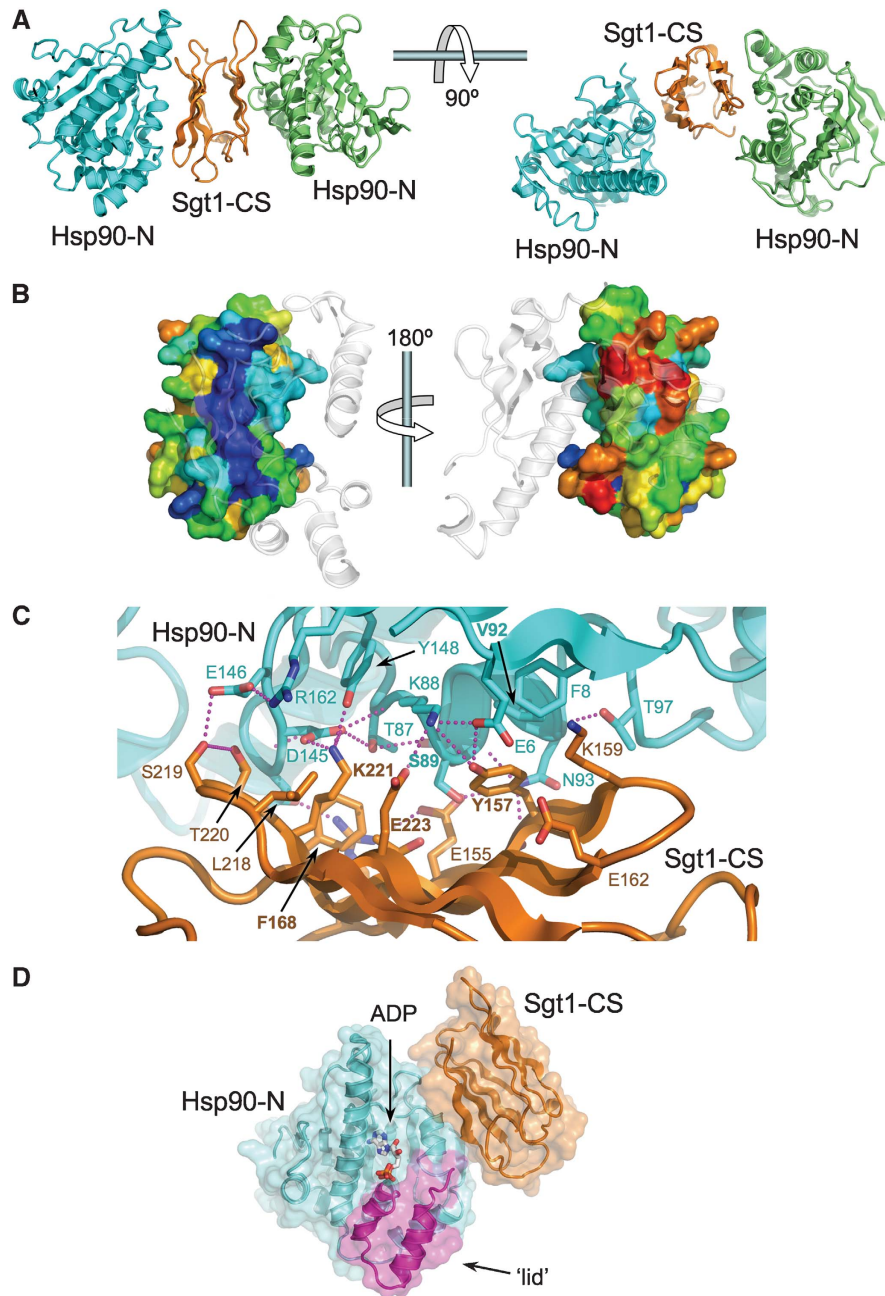
Hsp90-N–Sgt1-CS interface

The crystal lattice is such that each of the β-sheet faces of the Sgt1-CS domain structure contacts a different Hsp90-N domain through distinct regions (Figure 1A). Although a single Sgt1 molecule might interact simultaneously with both-N domains in an Hsp90 dimer, this is inconsistent with the ~1:1 binding stoichiometry suggested by ITC (Supplementary Figure 1). It is most likely that one of the Hsp90–Sgt1 interfaces is the biologically significant interaction, whereas the other is a lattice contact necessary for crystal formation. To identify the biologically significant interface, we analysed the conservation of the residues involved. In the case of Hsp90, which is highly conserved throughout, there was no clear difference between the two points of contact with Sgt1-CS in the crystal. However, Sgt1 showed a marked difference, with the residues involved in one interaction being very much more conserved overall, and with some contributing residues (Tyr157, Lys221 and Glu223) identical in all species (Figure 1B; Supplementary Figure 2).

The conserved interface is formed by residues from strands one, two and three from the four-stranded face of Sgt1-CS and the first strand and third helix of Hsp90-N domain (Figure 1C). The core interaction is provided by Tyr157 from AtSgt1a, the hydroxyl group of which hydrogen bonds to the side chains of Glu6 and Lys88 of HvHsp90, whereas its aromatic face packs into a hydrophobic recess on HvHsp90 formed by the side chains of Phe8, Val92 and Lys88, and the main chain of Ser89. Sgt1 Tyr157 is absolutely conserved, as are all the interacting residues in Hsp90, apart from Ser89, which is alanine in non-plant Hsp90s. Phe168 provides a second hydrophobic interaction, packing between the side chains of Thr87, Asp144 and Asp145 on Hsp90. Phe168 is also highly conserved, being phenylalanine in plants and yeast, and methionine in metazoa. These hydrophobic interactions are supported by an extensive network of polar interactions involving Arg153, Glu155, Thr166, Lys170, Lys221 and Glu223 from Sgt1, and Ser89, Asn93, Asp144, Asp145 and Glu146 from Hsp90 (Figure 1C). All the Sgt1 residues involved are at least very highly conserved, whereas the Hsp90 residues, with the exception of Ser89, are invariant. The Sgt1-CS interaction site on Hsp90 does not involve the mobile lid segment nor does it impinge upon the ATP-binding pocket at the heart of the N domain (Figure 1D).

Figure 1 Crystal structure of Hsp90-N-Sgt1-CS complex. (**A**) In the crystals, opposite faces of the Sgt1-CS domain β-sandwich structure interact with different parts of adjacent Hsp90-N domains. (**B**) Conservation colouring of the Sgt1-CS surface (blue→red; most→least conserved) based on the alignment in Supplementary Figure 2. The surface shown on the left is highly conserved with a ridge of essentially invariant residues that form the interface with Hsp90. The very high degree of conservation suggests that this interface is biologically authentic, whereas that on the right is a crystal lattice contact. (**C**) Detail of the conserved Hsp90-N–Sgt1-CS interface. The interface is built around two hydrophobic patches, centred on Sgt1-Tyr157 and Phe168, reinforced by a network of hydrogen bonding interactions (dotted lines). Formation of the complex buries ∼1100 Å² of molecular surface, which is consistent with a reversible interface. (**D**) Overview of the Hsp90-N–Sgt1-CS interaction based on the conserved interface. The ADP bound in the pocket of the Hsp90-N domain and the mobile 'lid' segment that closes in the ATP-bound state of Hsp90 are indicated.

Targeted disruption of Hsp90–Sgt1 interaction

Although the conservation pattern clearly identifies the biologically significant Hsp90–Sgt1 interface, we were concerned to verify this interaction outside the context of a crystal lattice. We examined the effect of mutations in residues on the surface of AtSgt1a-CS, on its ability to interact with the HvHsp90-N domain, using the Y2H system (Boter *et al*, 2007). Mutations of AtSgt1a-Tyr199, at the heart of the non-conserved crystal lattice contact, had no effect on the Y2H interaction with Hsp90, nor did mutation of AtSgt1a-Thr220

on the periphery of the conserved interface. However mutations of Tyr157, Phe168, Lys221 and Glu223, in the conserved interface, all significantly diminished the interaction, confirming its functional significance (Figure 2A). None of these mutations affected the Y2H interaction with AtRAR1, the interaction of which is mediated by a different region of the AtSgt1a-CS domain (Boter *et al*, 2007), confirming that these mutations do not disrupt CS domain folding.

To confirm that this interaction was conserved across species, we used an *in vitro* co-precipitation assay with
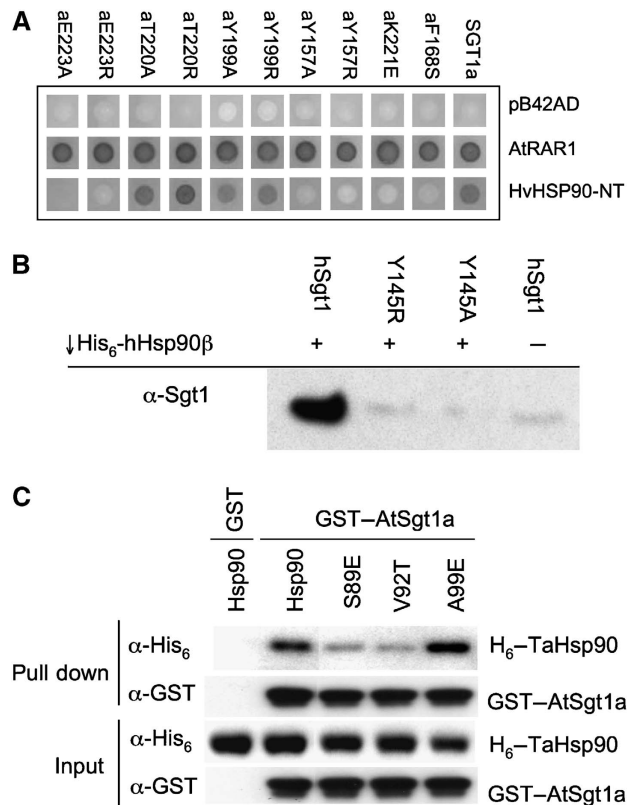
Figure 2 Targeted disruption of Hsp90-N–Sgt1-CS interaction. (**A**) Yeast two-hybrid analysis of AtSgt1a-CS mutants. AtSGT1a or its derivatives were assayed for interaction with AtRAR1 and HvHSP90-N using LexA Yeast Two-Hybrid system. Mutation of residues Tyr157, Phe168, Lys221 or Glu223, in the conserved interface with Hsp90-N, abrogated reporter activation. (**B**) Co-precipitation assay with His$_6$-tagged human Hsp90β, and human Sgt1. Hsp90 efficiently co-precipitates wild-type Sgt1, but not mutants of the core interface residue, Tyr145 (equivalent to Tyr157 in the plant protein). (**C**) Co-precipitation assay with GST-tagged *Arabidopsis* Sgt1a, and plant Hsp90. AtSgt1a co-precipitates wild-type TaHsp90 or TaHsp90 with mutation of a residue not in the observed interface, but does not efficiently co-precipitate TaHsp90 with mutations in interfacial residues Ser89 or Val92.

human homologues to follow Hsp90–Sgt1 interactions in solution (see Materials and methods), and compared the interaction of wild-type Sgt1 with mutants intended to disrupt the conserved Sgt1–Hsp90 interface. GST-tagged full-length wild-type human Sgt1 efficiently co-precipitated hHsp90β (Figure 2B). In contrast, Arg or Ala mutations of Tyr145 (equivalent to AtSgt1a-Tyr157), significantly decreased the ability of hSgt1 to co-precipitate hHsp90β.

Using the plant system, we mutated several residues in the wheat (*Triticum aestivum*) Hsp90-N domain that are involved in the interface with Sgt1, which also involves the equivalent of Tyr157. Mutation of TaHsp90 Ser89 and Val92, both of which directly contact AtSgt1a Tyr157 to Glu and Thr, respectively, significantly decreased their ability to be co-precipitated by GST–AtSgt1a when compared with wild type. By contrast an Ala99Glu mutant, which is not directly involved in this interface, was efficiently co-precipitated (Figure 2C).

Taken together, the strong conservation of the residues on the four-stranded face involved in interaction with Hsp90 and the disruption of that interaction, *in vitro* and in the Y2H system, by mutations on either side of that interface argue
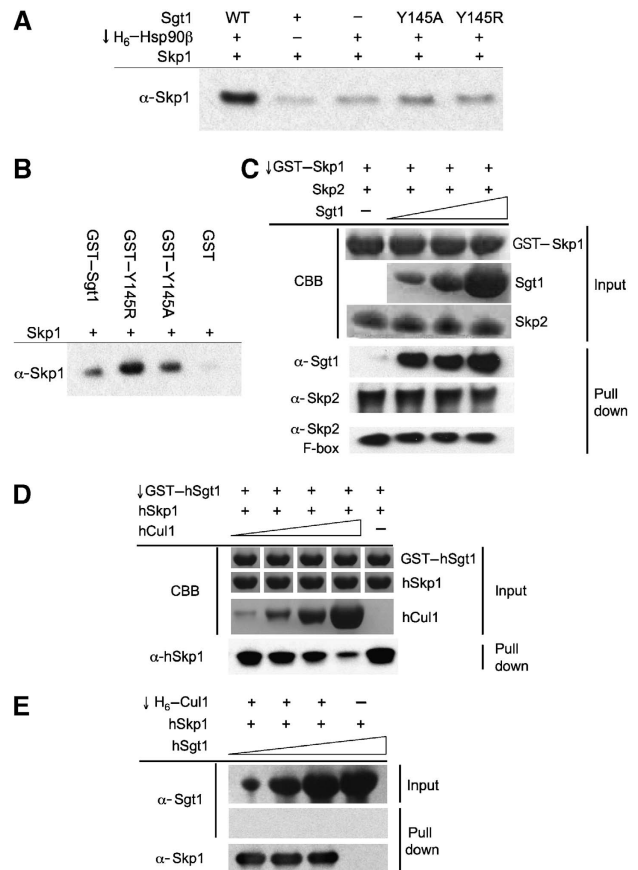
Figure 3 Sgt1 interactions with SCF complex. (**A**) Bridged co-precipitation assay with His$_6$-tagged human Hsp90β, Sgt1 and Skp1. Hsp90 efficiently co-precipitates Skp1 (visualized by western blot) only when wild-type Sgt1 is present to bridge the interaction. Mutations in the Sgt1 Hsp90-binding residue Tyr145 prevent Skp1 co-precipitation. (**B**) Direct co-precipitation of Skp1 by Sgt1 (visualized by western blot) is not affected by Sgt1 Tyr145 mutations. (**C**) Sgt1 is co-precipitated by GST–Skp1–Skp2 or GST–Skp1–Skp2 (F-Box) complexes, with no competitive displacement of Skp2 by increasing concentrations of Sgt1. Input protein loadings (10%) are visualized with Coomassie Brilliant Blue (CBB) and co-precipitated proteins are visualized by western blot. (**D**) Co-precipitation of Skp1 by GST-tagged Sgt1 is diminished by increasing concentrations of Cul1, showing competition between Sgt1 and Cul1 for binding to Skp1. Protein is visualized as in (C). (**E**) His$_6$-tagged Cul1 efficiently co-precipitates Skp1, but not Sgt1, showing that there is no direct interaction between Cul1 and Sgt1, and that Skp1 cannot bind Cul1 and Sgt1 simultaneously. Increasing concentrations of Sgt1 fail to displace Cul1, which binds Skp1 ~70-fold tighter than Sgt1.

very strongly that this is the biologically authentic site of interaction between the two proteins and that it is both necessary and sufficient for that interaction.

Sgt1 couples Hsp90 to SCF complexes

Yeast Sgt1p binds simultaneously to yHsp90 (Hsp82p) and ySkp1p, suggesting that it functions as an adaptor linking the two proteins (Catlett and Kaplan, 2006). We verified this interaction using the human proteins and observed co-precipitation of hSkp1 with His$_6$-tagged hHsp90α only in the presence of hSgt1 (Figure 3A). With the Tyr145Arg or Tyr145Ala hSgt1 mutants, no hSkp1 was co-precipitated. However, GST fusions of both hSgt1 mutants were fully competent in co-precipitating hSkp1, confirming that the Hsp90- and Skp1-interacting regions of Sgt1 are functionally independent (Figure 3B).

Skp1 is functionally associated with a variety of proteins containing an 'F-box'—a small helical-coil domain that mediates interaction with the C-terminal part of Skp1 in the context of SCF E3 ubiquitin ligases (Hao *et al*, 2005). Previous studies have shown that interaction with Skp1 is mediated by the TPR domain of Sgt1 (Catlett and Kaplan, 2006). As TPR domains are also helical-coil structures, we considered the possibility that Sgt1 might be a cryptic 'F-box' protein and interact with the same site on Skp1. To test this, we co-expressed human Skp1 with Skp2, an F-box protein that mediates the recruitment of p27^{kip1} to SCF complexes (Hao *et al*, 2005), which would therefore compete with Sgt1 if it interacted with the F-box-binding site on Skp1. We found that the Skp1–Skp2 complex or a Skp1–Skp2(F-box) complex was fully able to co-precipitate Sgt1, without competitive displacement of Skp2 with increasing concentrations of Sgt1 (Figure 3C). This suggests that Sgt1 interacts primarily with the N-terminal lobe of Skp1 and can coexist in Skp1 complexes with Skp2 (and probably other F-box proteins), the binding site of which is in the C-terminal lobe.

As well as binding F-box proteins, Skp1 binds to Cullin scaffold proteins such as Cul1, through its N-terminal lobe. Cullins couple Skp1 to the Rbx1 ring-finger protein that catalyses transfer of ubiquitin from an E2 ubiquitin conjugate, to a lysine on the target protein bound by the F-box protein, or to a ubiquitin molecule already attached to the target. Again, we considered the possibility that the TPR domain of Sgt1 might bind to Skp1 in competition with Cul1. Although GST–Sgt1 was able to co-precipitate Skp1 in isolation, the presence of Cul1 in the reaction decreased the co-precipitation of Skp1 in a dose-dependent manner (Figure 3D). In the inverse experiment, Cul1 co-precipitated Skp1, but not Sgt1 present in the same reaction (Figure 3E), confirming that Cul1 and Sgt1 do not interact directly, and that they bind to Skp1 with mutual exclusion. However, we were not able to decrease the amount of Skp1 co-precipitated by Cul1 with increasing amounts of Sgt1. To analyse this further, we directly determined the affinities of Sgt1 and of Cul1, for Skp1, using isothermal titration calorimetry (Supplementary Figure 3). Although Sgt1 bound in the low micromolar range ($K_D = 1.64\,\mu M$), Cul1 bound Skp1 $\sim$70-fold more tightly, with a dissociation constant of $\sim$24 nM, so that Cul1 could not be competed off a Cul1–Skp1 complex by comparable levels of Sgt1. Taken together, these data are fully consistent with Sgt1 and Cul1 binding in mutual exclusion at a common or at least overlapping binding site on the N-terminal half of Skp1.

In vivo coupling of Hsp90- and Sgt1-centred complexes

Hsp90 is the hub of a nexus of physical and functional interactions with complexes involved in signalling and transcriptional regulation, the component proteins of which form the dependent clientele of the Hsp90 chaperone system (Pearl and Prodromou, 2006; Zhao and Houry, 2007). Sgt1 has also been implicated in the assembly and function of diverse protein complexes, including CBF3 kinetochore, SCF E3 ubiquitin ligase and adenyl cyclase complexes in yeast (Kitagawa *et al*, 1999; Dubacq *et al*, 2002) and R-proteins and Nod-like receptors in plants and animals (Peart *et al*, 2002; Bieri *et al*, 2004; Leister *et al*, 2005; da Silva Correia *et al*, 2007; Mayor *et al*, 2007). Sgt1 therefore also has the characteristics of an interaction hub. However, it is not clear

whether Sgt1 has an independent function in any of these systems, or whether it functions purely as a scaffold protein, coupling them to Hsp90.

To gain some insight into this, we utilized our structural and biochemical data to engineer mutations in plant Sgt1, *in vivo*, which would be expected to specifically disrupt its interaction with Hsp90, but not with other known ligand proteins. Using a GFP-tagged fluorescent virus propagation assay, as described earlier (Azevedo *et al*, 2006; Boter *et al*, 2007), we determined the Rx-protein-mediated resistance to infection by potato virus X (PVX), of tobacco leaves (*Nicotiana benthamiana*) silenced for endogenous Sgt1, and heterologously complemented with wild-type or mutant AtSgt1a (see Materials and methods). Alanine mutations of Tyr199 on the three-stranded face of AtSgt1a-CS domain or of Thr220 on the four-stranded face, but not involved in direct contact with Hsp90, had no effect on the ability of the mutant protein to confer resistance to infection. Alanine mutations of Glu223 or Thr166, which are part of the Hsp90-interacting surface, also had little effect on resistance. However, alanine mutation of Tyr157, at the heart of the interface with Hsp90, or a charge reversal mutation of Lys221 close by, caused a substantial loss of resistance, comparable to an Sgt1a null (Figure 4A). The ability of a single missense mutation in Sgt1 that specifically disrupts its interaction with Hsp90 *in vitro*, to disrupt R-gene-mediated viral resistance *in vivo*, provides definitive evidence for the direct dependence of this class of innate immunity system on the Hsp90 chaperone system, with Sgt1 acting as a molecular scaffold coupling the two.

Although yeast lacks homologues of plant R-proteins or animal Nod-like receptors, Sgt1 is essential in yeast, not least due to its involvement in the CBF3 kinetochore complex, and the SCF E3 ubiquitin ligases that regulate cell cycle transitions (Kitagawa *et al*, 1999). To determine whether essential Sgt1 functions in yeast are also dependent on its interaction with Hsp90, we engineered similar interface-disrupting point mutations, singly and in combination, into yeast Sgt1 and determined their ability to confer viability in the absence of a wild-type Sgt1 allele (see Materials and methods). Unlike the plant system, single-point mutations in either of the main hydrophobic Sgt1 interface residues (Tyr190, Phe201≡Tyr157, Phe168 in plants) had little effect (Figure 4B). However, double mutants abrogated viability. This is consistent with the observation of multiple missense mutations in previously identified *ts SGT1* alleles (Kitagawa *et al*, 1999). Furthermore, although viable under normal conditions, yeast harbouring Sgt1 single-point mutants were significantly more sensitive to the Hsp90 inhibitor, geldanamycin, than wild type (Figure 4C). Taken together, these data confirm the dependence of essential Sgt1 functions in yeast on its ability to form a stable complex with Hsp90.

TPR co-chaperone compatibility

The extreme C terminus of Hsp90 consists of an MEEVD motif that provides the binding site for a range of co-chaperones containing TPR domains. Although Sgt1 possesses a TPR domain, this is not essential for Hsp90 binding (Lee *et al*, 2004; Lingelbach and Kaplan, 2004). Furthermore, a yeast TPR domain co-chaperone, Sti1 known to interact with the C terminus of Hsp90, bound simultaneously with Sgt1 (Catlett and Kaplan, 2006). We found that the human TPR domain co-chaperone CHIP was also able to bind to Hsp90 at the
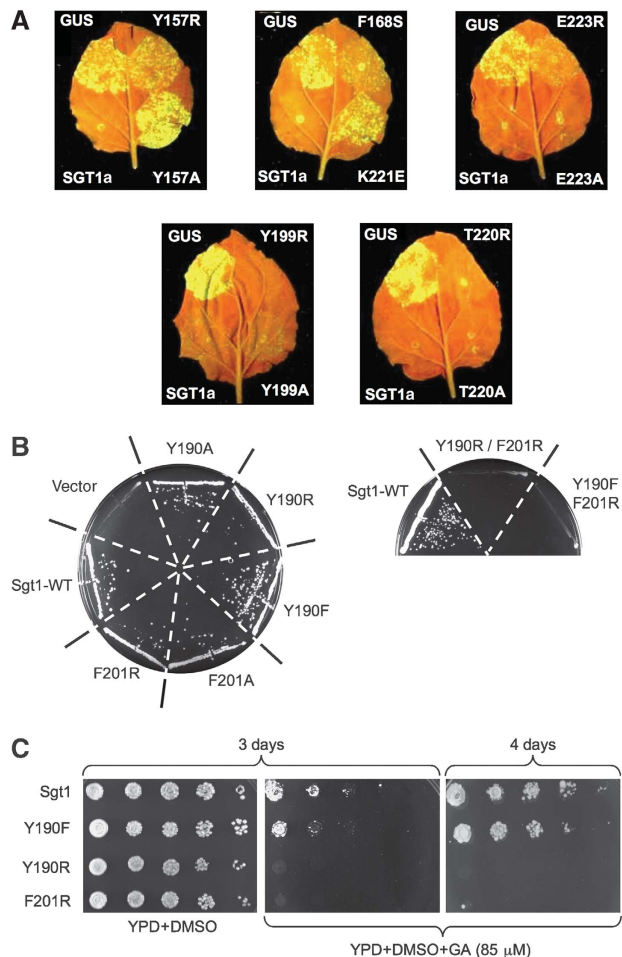
Figure 4 Functional dependence of Sgt1 on Hsp90 interaction *in vivo*. (**A**) Functional assay of SGT1a mutants in Rx-mediated resistance against potato virus X (PVX). *Rx*-containing *N. benthamiana* plants silenced for *NbSGT1* were co-infiltrated with *Agrobacterium* expressing wild-type *AtSGT1a* (lower left, positive control), or *AtSGT1a* mutants as indicated (right half of the leaf) or *GUS* (upper left, negative control) together with *PVX-GFP*. Virus accumulation was monitored by GFP fluorescence under UV illumination 5 days after inoculation. Mutations in residues not involved in the core interface (Tyr199 and Thr220) with Hsp90 had little effect on the ability of AtSgt1a to facilitate viral resistance. Mutations of the hydrophobic Hsp90 interface residues Tyr157 or Phe168, and charge-reversal mutations in polar interface residues Lys221 or Glu223, severely impaired the biological function of Sgt1a. (**B**) Single-point mutations in Hsp90-interacting residues did not significantly impair essential Sgt1 functions in yeast (left), but double mutants abolished viability (right). (**C**) Single-point mutations in Hsp90-interacting residues in yeast Sgt1 sensitize yeast to killing by the Hsp90 inhibitor geldanamycin (GA).

same time as Sgt1 (Figure 5), showing that Sgt1 can most likely coexist in Hsp90 complexes with a range of TPR domain co-chaperones. As Sgt1 is able to couple Hsp90 to the Skp1 and F-box components of SCF E3 ligase systems, its ability to coexist in Hsp90 complexes with CHIP, an active E3 ubiquitin ligase, is particularly interesting.

Discussion

### Hsp90 interaction and nucleotide dependence

The Hsp90–Sgt1 core complex structure reveals a previously unknown site of co-chaperone interaction on the chaperone. Binding sites have been characterized in the middle domain
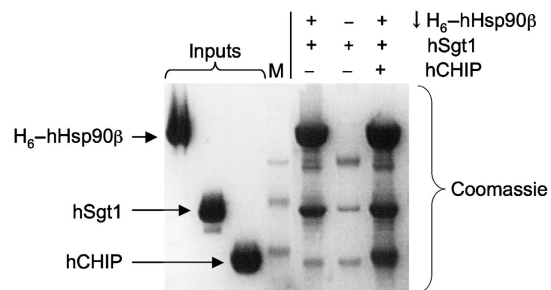
Figure 5 Co-binding of TPR domain E3 ligase CHIP and Sgt1. His$_6$-tagged human Hsp90β efficiently co-precipitates Sgt1 and CHIP, confirming that the TPR domain of Sgt1 does not interact with the C-terminal MEEVD sequence of Hsp90, which provides the binding site for CHIP, and other TPR domain co-chaperones.

for the ATPase activator Aha1 (Meyer *et al*, 2004), and at the C-terminal MEEVD motif for a variety of TPR domain co-chaperones (reviewed in Pearl and Prodromou, 2006). Sgt1 also possesses a TPR domain, but this does not bind to Hsp90 in isolation, and Hsp90 constructs lacking the MEEVD motif are still able to bind Sgt1 (Catlett and Kaplan, 2006). Cdc37 interacts with the open conformation of the lid segment in the N-terminal nucleotide-binding domain and arrests Hsp90's ATPase cycle (Roe *et al*, 2004). p23/Sba1 also interacts with the lid segment in the N domain, but only in its closed conformation within the closed ATP-bound state of the chaperone, and in doing so reinforces the interaction of the N domains within the closed dimer to each other and to the middle domains (Ali *et al*, 2006). Although the CS domain of Sgt1 and the globular core domain of p23/Sba1 have similar three-dimensional structures, they bind to different regions of the Hsp90 surface, using different parts of their own structures. Sgt1 utilizes residues on the surface of the four-stranded β-sheet, whereas a C-terminal strand, not present in Sgt1-CS, provides the majority of p23/Sba1a interaction with Hsp90-N (Figure 6A).

Most significant mechanistically are the different sites of interaction of p23/Sba1 and Sgt1-CS on Hsp90-N, and their effect on the conformational changes associated with Hsp90s ATPase cycle. p23/Sba1 interacts with residues on the lid segment of Hsp90 that are only available in the ATP-bound conformation of the chaperone (Ali *et al*, 2006), explaining the strongly ATP-dependent binding of p23/Sba1 (Sullivan *et al*, 1997; Siligardi *et al*, 2004). Sgt1-CS binds to Hsp90-N away from the lid and ATP-binding pocket, so that its preferential binding in the absence of ATP (Lee *et al*, 2004; Catlett and Kaplan, 2006) is difficult to explain at first sight. However, part of the interaction is provided by Glu6 and Phe8 from the N-terminal strand of Hsp90, which undergoes a large movement during the ATPase cycle, swapping from its own N domain in the open ATP-free state (Prodromou *et al*, 1997) to bind to the other N domain in the closed ATP-bound conformation (Ali *et al*, 2006), recapitulating its previous interactions. Although Sgt1-CS could interact with the N-terminal strand in the ATP-free or ATP-bound states, it would certainly be displaced during the ATP-driven conformational change, so that in the presence of ATP the equilibrium concentration of Hsp90–Sgt1 complexes would be lower than in its absence. Furthermore, when the Hsp90-N–Sgt1-CS complex is used to model binding of Sgt1-CS domains to both Hsp90-N domains in the closed ATP-bound
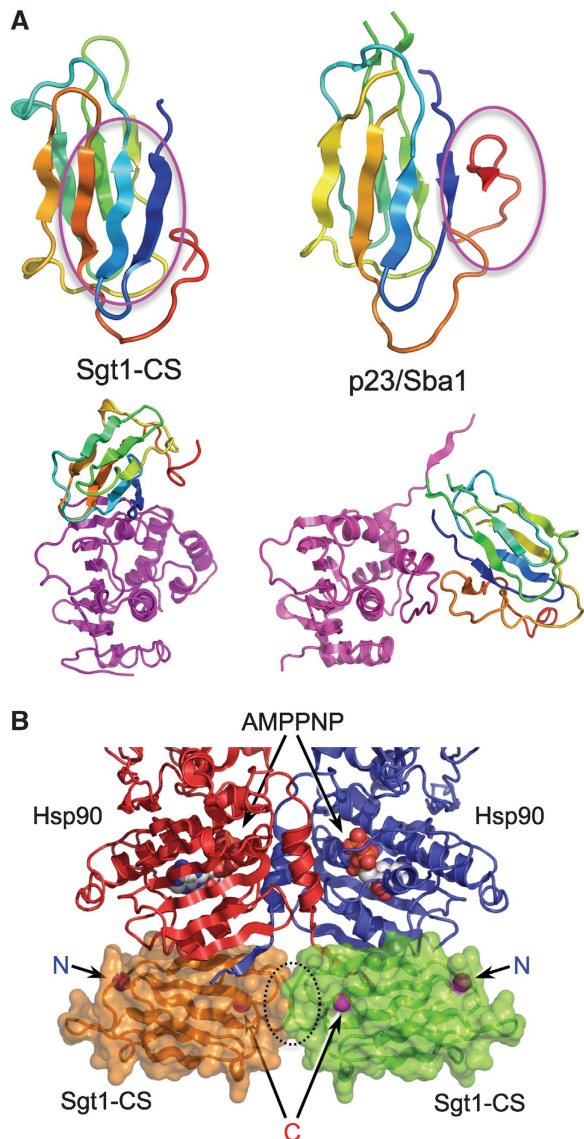
Figure 6 CS domain interactions. (**A**) Comparison of the binding surfaces used by the structurally homologous CS domains of the Hsp90 co-chaperones Sgt1 (left) and p23/Sba1 (right). Sgt1-CS uses residues on the face of its four-stranded β-sheet, whereas p23/Sba1 interacts with Hsp90-N through a C-terminal extension not present in Sgt1. The interaction site on Hsp90-N (bottom, magenta) is also completely different, with Sgt1-CS binding the side of the domain, whereas p23/Sba1 binds directly to the lid segment in its closed ATP-bound conformation. (**B**) Hypothetical model of Sgt1-CS binding to Hsp90 in the closed ATP-bound conformation, made by superimposing the Hsp90-N domain from the present structure on to the crystal structure of the full-length Hsp90-AMPPNP-p23/Sba1 structure (PDB code 2CG9). Consistent with the preference of Sgt1 for binding to the open ADP-bound state of Hsp90, the docked Sgt1-CS domains clash sterically, and this is likely to be exacerbated in the context of the full-length protein by the TPR and SGS domains, which extend from the N- and C terminus, respectively.

structure of the dimer (Figure 6B), there is a steric clash between the two Sgt1-CS domains. This may be exacerbated by the presence of the N-terminal TPR and C-terminal SGS domains, the disposition of which within the overall Hsp90–Sgt1 complex is not currently known.

Function of the Hsp90–Sgt1 couple

The range of functions in which Hsp90 is an essential participant continues to increase (Pearl *et al*, 2008). Rather

than clarifying the biochemical basis of Hsp90 involvement, the diversity of the expanding protein clientele makes it increasingly difficult to understand selective interaction with Hsp90 in terms of any common structural motif. At least part of this selectivity comes from co-chaperones such as Cdc37 that function as adaptors, recruiting specific classes of clients to Hsp90.

Sgt1 fulfils the requirements of an Hsp90 adaptor co-chaperone, interacting with Hsp90 through its CS domain, and with a range of putative client proteins through its TPR and SGS domains. Unlike Cdc37 however, it does not appear to regulate the ATPase activity of Hsp90 (Catlett and Kaplan, 2006). In plant innate immunity systems, Sgt1 is essential not only for the activity of R-proteins but also for their stable accumulation (Bieri *et al*, 2004; Azevedo *et al*, 2006; Boter *et al*, 2007). However, the related mammalian Nod-like receptors lose activity, but not stability, when Sgt1 is knocked down (da Silva Correia *et al*, 2007). They are nonetheless depleted by pharmacological inhibition of Hsp90, suggesting that this biologically widespread family of proteins are *bone fide* Hsp90 clients.

Chaperone–client relationships are less clear in the Sgt1-mediated coupling of Hsp90 and SCF complexes. A variety of different SCF subassemblies can be expressed in a functional state in bacteria, which argues against a stringent client dependence on Hsp90, and no depletion of SCF components on Hsp90 inhibition has been reported. Sgt1 physically couples Hsp90 and Skp1 (Catlett and Kaplan, 2006) and we have extended that observation to show that Sgt1 binds Skp1 in the presence of an F-box protein. Thus, Sgt1 links Hsp90 to the target-specific 'arm' of an SCF complex. The ability of Sgt1 to bind Skp1 alongside an F-box protein explains the association of Hsp90–Sgt1 with the yeast CBF3 kinetochore complex (Kitagawa *et al*, 1999; Stemmann *et al*, 2002; Bansal *et al*, 2004; Lingelbach and Kaplan, 2004; Rodrigo-Brenni *et al*, 2004; Steensgaard *et al*, 2004), as the Ctf13 component of CBF3 is an F-box protein, that is itself the target of ubiquitin-dependent degradation (Kaplan *et al*, 1997).

In contrast to F-box proteins, we found that Sgt1 binding to Skp1 was mutually exclusive with binding of Cul1, which provides the generic arm of the SCF complex, recruiting the ring-finger E3 ubiquitin ligase enzyme Rbx1. At first sight, this appears to be in contradiction to a reported co-precipitation from yeast lysates of the cullin Cdc53p, as well as Skp1p, by Sgt1p (Kitagawa *et al*, 1999). However, when SCF components were expressed in insect cells in the same study, the amount of Sgt1p co-precipitated by the F-box protein Cdc4p was decreased when Cdc53p was also present, consistent with our clear observation using purified human proteins that Cul1 and Sgt1 compete for Skp1 binding.

Association of the cullin arm with the specific arm of SCF complexes, through binding to Skp1, is regulated by a complex cycle of reversible modification involving covalent attachment of NEDD8, a ubiquitin-like protein, to Cul1. Neddylated Cul1 in complex with Rbx1 associates with Skp1 and its attendant F-box protein and is fully competent to recruit E2 ubiquitin conjugates and ubiquitinate the substrate protein bound to the F-box protein (Wu *et al*, 2006). This active SCF complex is inactivated when NEDD8 is removed by the COP9 signalosome complex (Lyapina *et al*, 2001). De-neddylated Cul1 is then bound by Cand1 with concomitant displacement of the Skp1–F-Box subcomplex
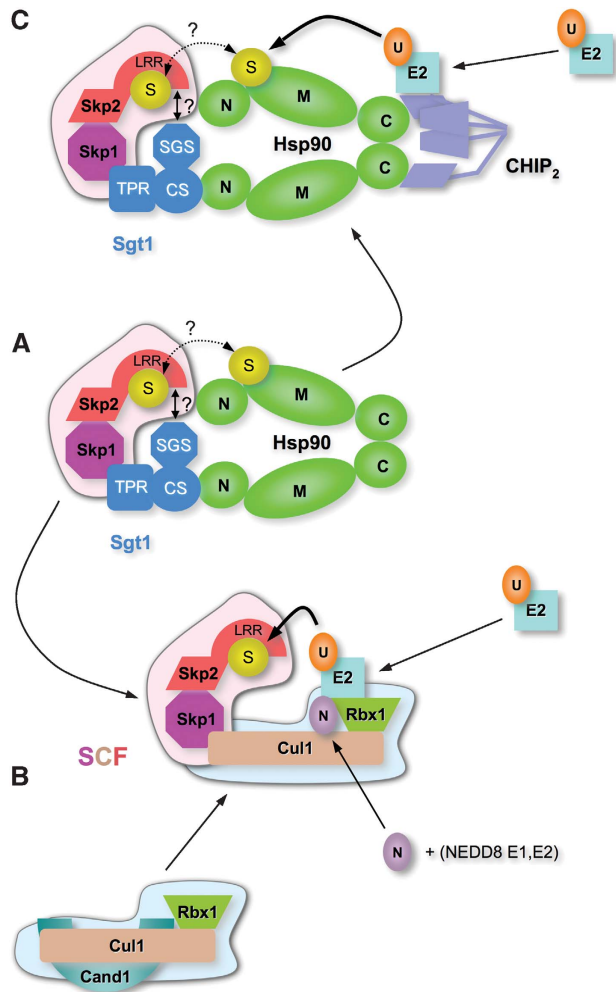
Figure 7 A role for Hsp90–Sgt1 in SCF assembly. (**A**) Previous observations and data presented here that Hsp90–Sgt1 can interact with Skp1 in complex with an F-box protein such as Skp2. In such a complex, a client/substrate protein (S) could be bound to either the chaperone or to the F-box protein, and in principle could be transferred from one to the other. (**B**) Competitive displacement of Hsp90–Sgt1 from Skp1 by Cul1–Rbx1, concomitant with the release of Cul1–Rbx1 from Cand1, would permit formation of an active SCF complex, able to be neddylated and bind E2. Disruption of the Cand1–Cul1 interaction might require the ATPase activity of Hsp90. (**C**) Alternatively, as a parallel pathway during stress conditions or when the SCF route is overloaded, an Hsp90–Sgt1–Skp1–F-box protein complex could allow ubiquitination of a client/substrate protein by the recruitment of CHIP to the C-terminal binding site on Hsp90.

and blockade of the NEDD8 attachment site on the cullin (Goldenberg *et al*, 2004). Regeneration of an active SCF complex thus requires displacement of Cand1, neddylation and association with a new Skp1–F-Box substrate complex (Wu *et al*, 2006). The ability of Sgt1 to couple Hsp90 to the specific arm of SCF complexes, along with its competitive displacement by the cullin arm, suggests a clear chaperone role for Hsp90–Sgt1 in binding and stabilizing a Skp1–F-Box substrate complex in the absence of Cul1–Rbx1. This is fully consistent with the observation of the ~70-fold higher affinity we observe with Cul1, as compared with Sgt1, for binding to Skp1, which would ensure efficient displacement of Hsp90–Sgt1 from a new Skp1–F-Box substrate complex by Cul1, once freed of its inhibitory association with Cand1. The

ATPase cycle of Hsp90 could have a function in facilitating the release of Cul1 from Cand1, and allowing its re-association with Skp1 to regenerate an active SCF complex (Figure 7A and B); however, further work will be required to test this possibility. A role for Hsp90–Sgt1 in facilitating the assembly of SCF complexes is fully consistent with observations that defective *SGT1* alleles in yeast impair SCF-dependent turnover of Sic1p *in vivo*, and extracts from *SGT1*-impaired strains are defective in cyclin ubiquitination *in vitro* (Kitagawa *et al*, 1999).

Hsp90 might also have a function in facilitating or regulating access of the substrate protein to the SCF system. A number of proteins known to be Hsp90 clients are degraded through SCF complexes, most significant of which is probably the cell cycle regulatory tyrosine kinase Wee1 (Aligue *et al*, 1994; Watanabe *et al*, 2004). However, the interplay between these two Wee1-interacting pathways has not so far been characterized, although both are required for proper mitotic entry. It has long been known that inhibition of Hsp90 promotes ubiquitin-dependent degradation of its client proteins (Mimnaugh *et al*, 1996), but the E3 systems responsible, particularly for protein kinases, have not been unambiguously identified. The TPR domain U-box protein CHIP is an E3 ubiquitin ligase able to associate with Hsp90 or Hsp70 through the C-terminal EEVD motif (Cyr *et al*, 2002; Zhang *et al*, 2005), and is a clear candidate for that role, although there are only a few clear demonstrations of CHIP's involvement in the degradation of major Hsp90 clients such as protein kinases (Xu *et al*, 2002; Zhang *et al*, 2007). The ability of CHIP to bind to an Hsp90–Sgt1 complex raises the possibility that it could have a function in ubiquitination of Skp1–F-Box-bound substrate proteins, possibly as a backup under stress conditions, or in some circumstances in direct collaboration with the SCF system (Nelson *et al*, 2006).

Materials and methods

Mutagenesis and plasmid construction

Amino-acid changes were generated in the *SGT1* gene of the centromeric (single copy) *LEU2* plasmid pRS315-SGT1 (Lingelbach and Kaplan, 2004), using the QuickChange site-directed mutagenesis kit (Stratagene). The following mutations were introduced and confirmed by sequencing: Y190A, Y190R, Y90F (TAT to GCT, AGA and TTT, respectively); F201A, F201R (TTT to GCT and AGA, respectively) and the double mutants Y190R/F201R and Y190F/F201R.

Media and genetic techniques

The *S. cerevisiae* strain YKK39 was transformed with pRS315-SGT1 bearing wild-type or mutant *SGT1*, as indicated in figure legends (Rose *et al*, 1990). Transformants were selected on drop-out media without uracil and leucine. The ability of *SGT1* mutants to maintain cell viability was assessed by curing for pRS316-SGT1 by incubating the transformants on drop-out media without leucine, but containing uracil (25 mg/l) and 5-FOA (0.1%).

DNA constructs

cDNAs for *H. vulgare* Hsp90 and *A. thaliana* Sgt1a were as described (Boter *et al*, 2007). Human Hsp90β was as described (Panaretou *et al*, 2002). Human Skp1, Skp2, Sgt1 and Cul1 were amplified from IMAGE ESTs (4 243 711, 2 962 938, 2 985 858 and 40 118 923, respectively). Different constructs of Skp1 (residues 1–163, 1–70, 81–163 and Δ71–80) and Sgt1 were cloned into pGEX6P-1, Skp2 constructs (residues 1–411 and 89–141) were cloned into pET28a. Cul1 coding region was split into two halves (residues 1–410 and 411–776) as described earlier (Zheng *et al*, 2002) and cloned into pST38 (Tan, 2001) for co-expression.

Protein expression and purification

Plant and mammalian Hsp90 and Sgt1 constructs were all expressed in the *Escherichia coli* strain Rosetta (DE3) pLysS. Hsp90 constructs, were purified as described earlier (Prodromou *et al*, 1997). GST-tagged Sgt1 constructs were purified by ion exchange on Q-Sepharose and immobilized on glutathione Sepharose beads. Sgt1 was then released by PreScission cleavage and further purified on a Superdex 75 PG gel-filtration column. Human Skp1, Skp2 and Cul1 constructs were all purified as described earlier (Zheng *et al*, 2002).

In vitro protein co-precipitation assay

In total, 50 μg of each indicated protein (except protein in gradient titrations) was incubated in 50 μl glutathione Sepharose resin or Talon™ metal affinity resin in 200 μl of binding buffer consisting of 50 mM Tris pH 7.5, 150 mM NaCl, 0.1% NP-40 and 1 mg/ml BSA (pH 7.5). For gradient titration experiments, 5 μg of gradient protein

was used in the first reaction, with three-fold increase in subsequent reactions (i.e. 15, 45 μg and so on). Reactions were performed overnight at 4°C. Resin was washed three times with 1 ml binding buffer later. One-fiftieth of the washed resin was resolved by SDS–PAGE, electroblotted and probed with antibody to the particular protein construct ('α-') as indicated. Quantities of protein consumed were shown by SDS–PAGE using 10% of each input proteins and visualized with Coomassie Brilliant Blue.

Crystallization, data collection and structure determination

HvHsp90-N and AtSgt1a-CS were combined in a 1:1 molar ratio in the presence of 5 mM ADP, incubated for 30 min and concentrated to 10 mg/ml by ultrafiltration. Initial multiple crystals were grown by vapour diffusion at 4°C against 26% w/v PEG4000, 100 mM Tris (pH 8.5) and 200 mM magnesium sulphate. Subsequent streak seeding into solutions of 16% w/v PEG4000, 100 mM Tris (pH 8.5) and 200 mM magnesium sulphate produced single thin plates. Crystals were harvested into reservoir solution with addition of glycerol (20% v/v) before flash cooling to 100 K. X-ray data were collected on beamline ID23 at the ESRF, Grenoble, from single crystals, and processed by using MOSFLM (Leslie, 1995) and the CCP4 package (CCP4, 1994). Crystals had space group $P2_12_12_1$, with three copies of the complex in the asymmetric unit. The thin plate crystals have a high solvent content (59% v/v) and only gave useful diffraction to 3.3 Å. The structure was solved by molecular replacement with Phaser (McCoy, 2007) using ADP-bound yeast Hsp90-N (PDB code: 1AMW) and the solution structure of human Sgt1-CS domain (PDB code: 1RL1) as search models, built using COOT (Emsley and Cowtan, 2004), and refined with CNS in PHENIX (Adams *et al*, 2002), using NCS restraints. Data collection and refinement parameters are given in Table I. Crystallographic data have been deposited in the Protein Databank with code: 2JKI

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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