Hop/Sti1 phosphorylation inhibits its co-chaperone function

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Abstract

In eukaryotes, the molecular chaperones Hsp90 and Hsp70 are connected via the co-chaperone Sti1/Hop, which allows transfer of clients. Here, we show that the basic functions of yeast Sti1 and human Hop are conserved. These include the simultaneous binding of Hsp90 and Hsp70, the inhibition of the ATPase activity of Hsp90, and the ability to support client activation *in vivo*. Importantly, we reveal that both Hop and Sti1 are subject to inhibitory phosphory-lation, although the sites modified and the influence of regulatory phosphorylation is species specific. Phospho-mimetic variants have a reduced ability to activate clients *in vivo* and different affinity for Hsp70. Hop is more tightly regulated, as phosphorylation affects also the interaction with Hsp90 and induces structural rearrangements in the core part of the protein.

Introduction

Hsp90 and Hsp70 are the most ubiquitously expressed molecular chaperones in eukaryotes and a central element of the folding machinery. Hsp70 interacts promiscuously with many different unfolded polypeptides already during translation but also generally prevents aggregation and supports refolding of aggregated and misfolded proteins [1]. In principle, this is achieved by recognizing and stabilizing exposed hydrophobic patches of substrates in an ATP-dependent manner [2]. In contrast, Hsp90 serves a more specific set of clients [3] and is mainly involved in a later stage of activation [4]. Among the clients of Hsp90 are many key regulatory proteins involved in signaling pathways, such as kinases and tran-scription factors [5,6]. These clients are structurally highly diverse, and co-chaperones are important for the interaction of Hsp90 with different clients [7]. Different co-chaperones bind to Hsp90 in a progressive manner, each stabilizing a specific conformation of Hsp90 and directing the conformational cycle which allows client activation [8].

For some clients, Hsp90 directly takes over substrates from Hsp70 [9]. The co-chaperone Sti1/Hop serves as an adaptor in this process as it physically connects the two chaperones and enables the transfer of clients [10]. Interestingly, Sti1/Hop is one of the few co-chaperones substantially induced by stress [11] suggesting its importance for Hsp90 both under physiological and stress condi-tions. Like Hsp90 and Hsp70, Hop is overexpressed in different cancer cells and promotes the progression of cancer [12]. This makes the interaction of Hop with Hsp90 and Hsp70 an interesting drug target [13–16].

A common interface for the interaction of co-chaperones with Hsp90 and Hsp70 is the TPR (tetratricopeptide) domain, defined by tandem repeats of a 34 amino acid consensus motif which forms a cleft of 7 antiparallel alpha-helices [17], capable of binding the C-terminal tails of the chaperones, both of which end in EEVD [18,19]. Sti1/Hop is a monomeric protein [20] consisting of three TPR domains, two DP (aspartate and proline rich) domains, and a flexible linker between TPR1–DP1 and TPR2A–TPR2B–DP2 (Fig 1A).

The structures of these domains have been solved recently [21,22]. While TPR1 and TPR2B are responsible for recognizing Hsp70, TPR2A specifically interacts with Hsp90 and the DP domains seem to contribute to substrate activation [21,23].

Yeast Sti1 is a potent non-competitive inhibitor of the Hsp90 ATPase activity [24,25]. The inhibition is achieved by binding of the central element of Sti1, TPR2A–TPR2B, to the C-terminal end of Hsp90 and the Hsp90 middle domain which leads to a stabilization of the open conformation of Hsp90 [21,25–27]. While the function of most Hsp90 co-chaperones is conserved between yeast and man, Sti1 and its mammalian homolog Hop seem to be different. It has been speculated that the mammalian protein Hop may affect the conformation of Hsp90 in a different manner compared to Sti1 [28–30]. Further, both proteins appear to be heavily phosphorylated, but potentially at distinct sites [11,31,32]. Therefore, we set out to analyze and compare the function of the Sti1/Hop homologs from yeast and human especially their regulation via phosphorylation.

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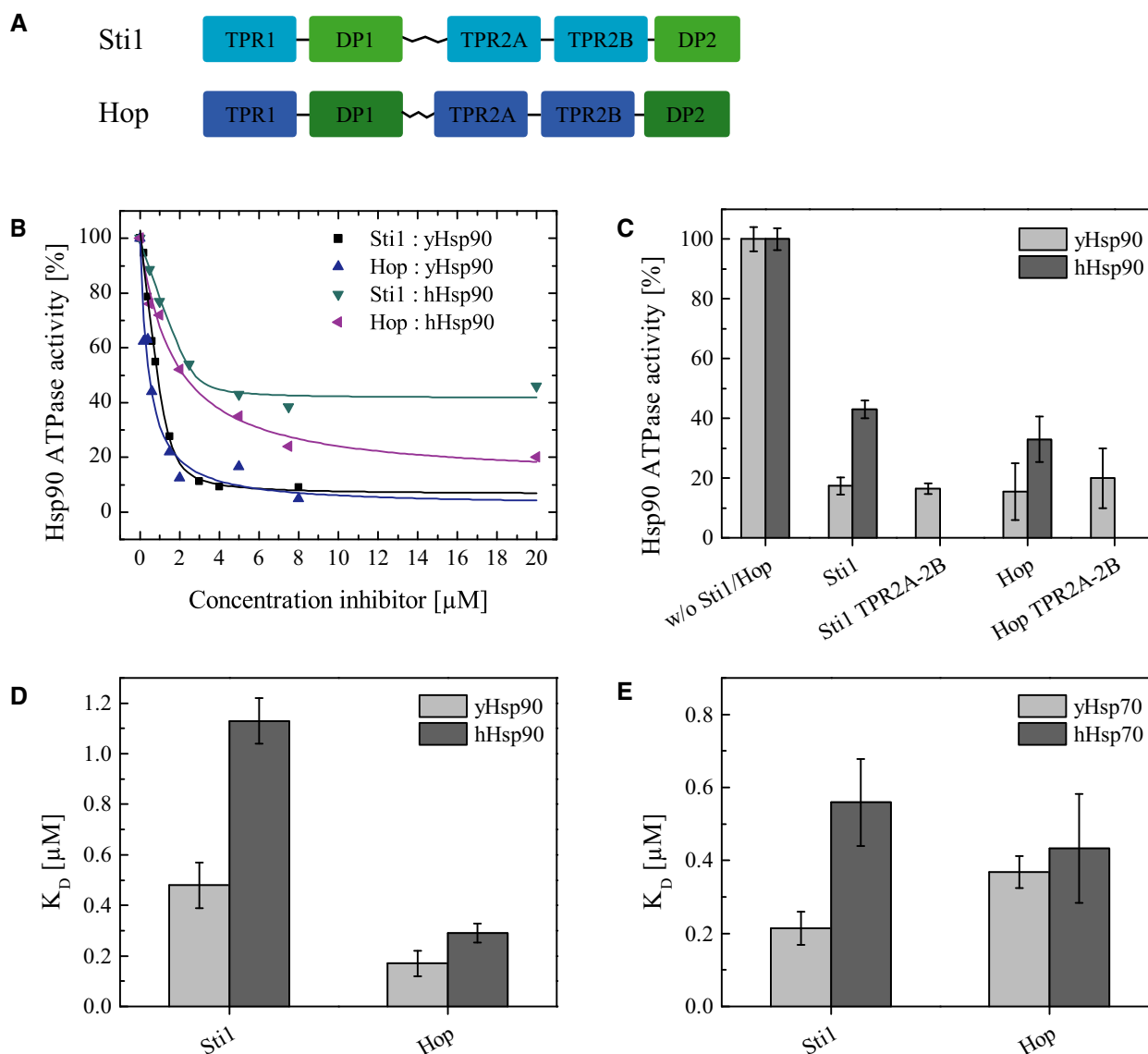
Figure 1. Sti1 and Hop interact similarly with Hsp90 and Hsp70.

A   Scheme of Sti1/Hop domains.

B   ATPase activity of y/hHsp90 upon addition of Sti1/Hop measured with an ATP-regenerative ATPase assay. 2 μM yHsp90 (30°C) or 5 μM hHsp90 (37°C) was used with varying amounts of inhibitor. Absolute values without inhibitor were k_{cat} of hHsp90: 0.23/min and k_{cat} of yHsp90: 0.88/min. Means of three independent measurements are shown.

C   Values at equimolar concentrations of Hsp90 and inhibitor for different combinations. Means of three independent measurements with the standard error are shown.

D, E Binding affinities of Sti1 and Hop toward Hsp90/Hsp70 homologs determined by surface plasmon resonance spectroscopy (SPR) using Hsp90- (D) or Hsp70-coupled (E) chips (Supplementary Fig S2). Error bars indicate standard error of the fit using titration data.

Results

Hop is a potent inhibitor of the Hsp90 ATPase activity

Sti1/Hop homologs share strong sequence similarity and comprise the same domain arrangement of two TPR-DP segments connect by a linker (Fig 1A) which exhibits the lowest degree of conservation (Supplementary Fig S1). In different yeast species, the length of the linker is conserved to around 60 amino acids. In contrast, the mammalian homologs exhibit a much shorter linker of around 30 amino acids with considerable sequence conservation (Supplementary Fig S1). Despite the high similarity of the Hsp90-interacting module TPR2A–TPR2B of yeast Sti1 and human Hop, different effects on the ATPase activity of Hsp90 have been reported [24,28]. To analyze these differences, we tested the inhibitory potential of Sti1 and Hop in authentic and mixed systems (Fig 1B and C). To our surprise, both Hop and Sti1 inhibited the ATPase activity of yHsp90 and hHsp90 substantially. For each combination, maximum inhibition was already reached at an equimolar concentration of dimeric Hsp90 and monomeric co-chaperone. Differences were

revealed regarding the Hsp90 homologs: The ATPase activity of yHsp90 was almost completely inhibited by Sti1 and by Hop (to about 15% of the basal activity), whereas the ATPase activity of hHsp90 was inhibited to a smaller extent (to about 30–40% of the basal activity). For both Sti1 and Hop, a shortened construct comprising only the central element TPR2A–TPR2B was already sufficient for inhibition of the Hsp90 ATPase activity highlighting that the minimal fragment responsible for the inhibition is conserved between yeast and man (Fig 1C). We further compared the affinities of the different combinations of Sti1/Hop with Hsp90 by surface plasmon resonance spectroscopy (SPR) (Supplementary Fig S2). The dissociation constants were determined to be in the same order of magnitude, although yHsp90 bound about twice as strongly to Sti1 and slightly stronger to Hop than its human counterpart (Fig 1D). Together, this indicates that the mechanism of Hsp90 interaction and regulation is conserved for Sti1 and its mammalian counterpart Hop, despite small differences in affinities and inhibition potentials.

We next tested the interaction of Hsp70 homologs with Sti1/Hop for different combinations. Hop and Sti1 exhibited similar affinity toward yHsp70 and hHsp70. In both cases, yHsp70 binds slightly stronger than human Hsp70 (Fig 1E). We also compared the homologs concerning their ability to form ternary complexes with Hsp90 and Hsp70 by analytical ultracentrifugation. For the yeast Hsp90–Sti1–Hsp70 complex, a detailed analysis of this complex had already been performed in the past [21]. Like Sti1 and Sti1–TPR2A–TPR2B, Hop or Hop–TPR2A–TPR2B were able to form ternary complexes with hHsp90 and hHsp70 as deduced by the peak shift from 5 S to 10 S and to 8.5 S, respectively (Fig 2A and B). This suggests that in both Sti1 and Hop, the central TPR2A–TPR2B element is the platform sufficient for the simultaneous binding of both chaperones and the key component of the adaptor.

Effects of Sti1 and Hop domains on maturation of the glucocorticoid receptor

The similarity of Hop and Sti1 *in vitro* prompted us to test their impact on an Hsp90 substrate protein in an *in vivo* context. For this, we measured the effect of Sti1/Hop variants on the activation of the glucocorticoid receptor (GR) in *sti1Δ* yeast cells which harbored a β-galactosidase-based reporter system. GR activity in yeast depends on an active Hsp90 system and the deletion of Sti1 drastically reduces GR activity [33]. In accordance with previous findings [34], Hop was able to compensate for the loss of Sti1 in the GR assay in *sti1Δ* yeast cells. When we tested the effect of single domains or fragments of Hop, we found that most were not able to support GR activation (Fig 2C). Only the Hop fragments ΔTPR1 and TPR2A–TPR2B–DP2 appreciably compensated for the loss of Sti1. This is reminiscent of the effect of individual Sti1 fragments [21]. Interestingly, this shows that in both homologs, apparently the TPR1–DP1 domain might be at least partially dispensable. It further shows that the yeast homolog Sti1 can be functionally replaced by the human protein Hop *in vivo*. However, Hop-ΔTPR1 (80%) was not as effective as Sti1-ΔTPR1 (100%), which indicates that species-specific differences might exist in the N-terminal part of Sti1/Hop. It also supports a functional contribution of the N-terminal TPR-domain, which may not be essential, but could be required under certain conditions.

Sti1 and Hop are regulated via phosphorylation at unique sites

Recently, a global phosphorylation screen of yeast proteins has identified six phosphorylation sites in Sti1 (T37, S95, S227, S258, S410, and T526) [31] (Fig 3A). For different mammalian Hop homologs, phosphorylation has been reported at five different sites: S16, S189, T198, Y354, and S481 (www.phosphosite.org) (Fig 3B). Phosphorylation at S189 and T198 in mouse Hop seems to control nuclear localization of the protein [32,35,36], but the other regulatory sites have not been analyzed yet.

For both Sti1 and Hop, the phosphorylation sites are found either in or directly flanking the Hsp70 binding domains, TPR1 and TPR2B, or in the linker connecting the main modules of Sti1/Hop (Fig 3A and B), while the Hsp90-interacting domains apparently are not regulated by phosphorylation. Except for S95 in Sti1, the phosphorylation sites are located in loop regions of the TPR domains or in unfolded segments. The specific sites modulated, however, are unique for Hop and Sti1. The only exception is the phosphorylation site S481 in Hop and T526 in Sti1, respectively, which are located at the same position in the sequence alignment (Supplementary Fig S1).

To analyze the effect of phosphorylation on Sti1/Hop, we generated glutamate-based phospho-mimicking variants of Sti1/Hop and introduced them into *sti1Δ* yeast cells to assess *in vivo* GR activation (Fig 3C and D). In the case of Sti1, all phospho-mimicking replacements lead to a reduction of GR activation. The weakest effect, a 30% reduction of GR activity was seen for S95E. The other variants exhibited a reduction between 50% and full suppression of chaperone-mediated GR activation. The strongest effects were caused by the mutations T37E and S410E. These are located in TPR1 and TPR2B, the two Hsp70 binding sites of Sti1 implicating a regulatory role for the interaction with Hsp70. The combination of different phosphorylation sites did not lead to an additive effect as a Sti1 construct comprising all phospho-mimicking mutations (Sti1 All-E) did not reduce GR activation more than the single glutamate mutants. Control alanine substitutions of Sti1 did not lead to a decrease of GR activation except for S95A (Fig 3E). For Hop, we choose to analyze the previously uncharacterized phosphorylation sites S16, Y354, and S481. In particular, Y354 is interesting as this site is located directly at the domain contacts between TPR2A and TPR2B, a region which is of crucial importance for the arrangement of the two domains and thus also for GR activation [21]. Interestingly, also the Hop phospho-mimics showed pronounced reduction in GR activity. While GR activity for Y354E was moderately reduced, S16E and S481E resulted in about 50% of wild-type activity. Control alanine/phenylalanine substitutions of Hop did not affect GR activation (Fig 3F).

Taken together, phosphorylation of Sti1 and Hop at different positions allows inhibiting the function of this co-chaperone *in vivo*. For both Sti1 and Hop, different degrees of regulation were observed.

Interaction of phospho-mimetic variants with Hsp90 and Hsp70 *in vitro*

The structural analysis of the phospho-mimics *in vitro* by CD spectroscopy revealed that the secondary structure or stability was not influenced. Also, small angle X-ray scattering (SAXS) analysis detected only minor shape differences of the phospho-mimicking
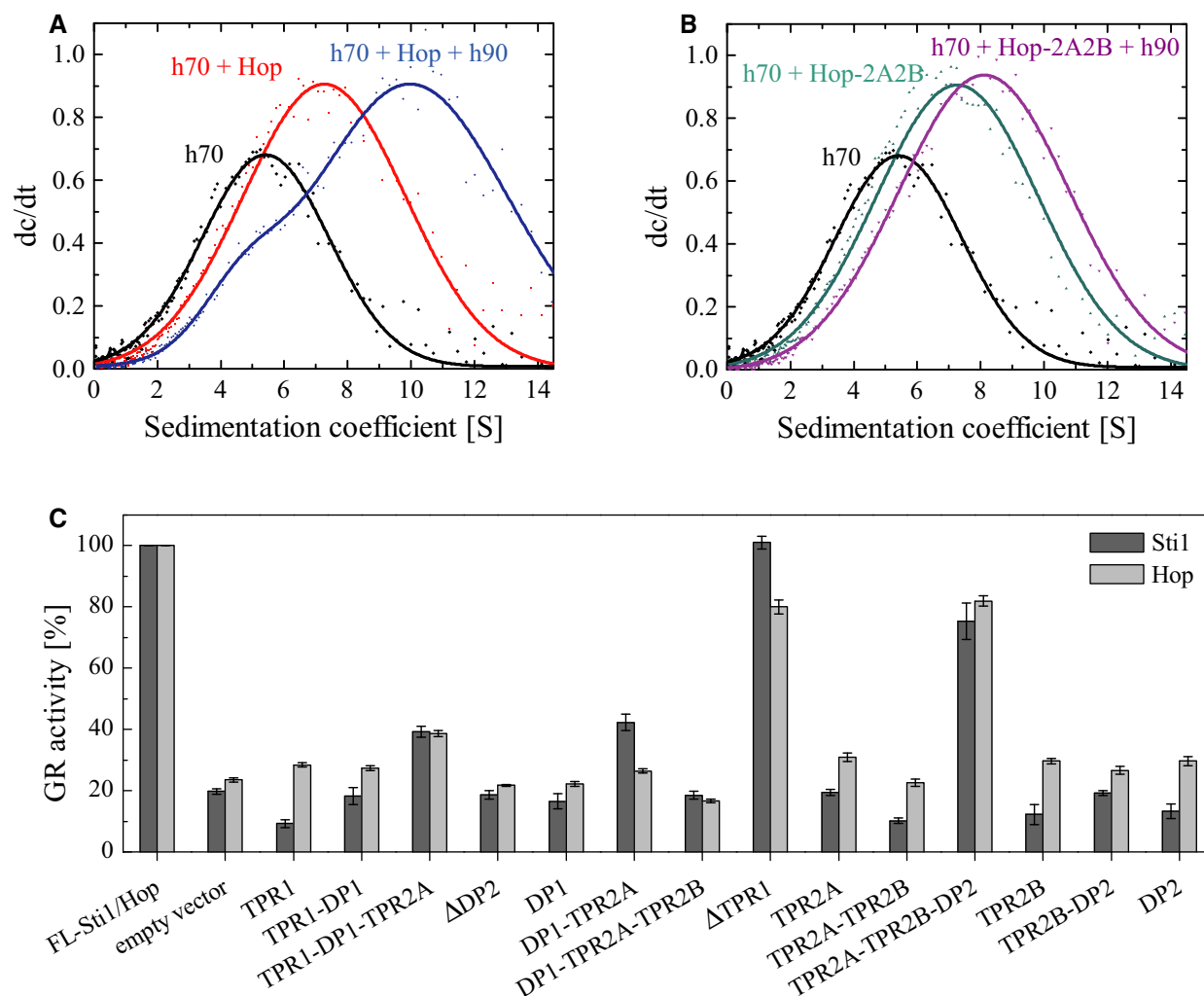
Figure 2. Sti1 and Hop are similar concerning ternary complex formation and *in vivo* activity.

A Formation of complexes of Hop with hHsp70 and hHsp90. Of 500 nM labeled hHsp70 alone (black), after addition of 3 μM Hop (red) and ternary complexes with 3 μM Hop and 3 μM hHsp90 (blue). The data were fitted to (bi-)Gaussian functions.

B Formation of complexes of Hop TPR2A–TPR2B with hHsp70 and hHsp90. Of 500 nM labeled hHsp70 in the presence of 3 μM Hop TPR2A–TPR2B (dark cyan) and ternary complexes after addition of 3 μM hHsp90 (magenta). The data were fitted to (bi-)Gaussian functions.

C GR activation measured with a β-galactosidase-based assay in *sti1Δ* yeast cells expressing different Sti1/Hop fragments. Means of three independent experiments are shown. Error bars indicate standard errors.

Sti1 variants in comparison with the wild-type protein (Fig 4A and Supplementary Table S1). However, the phospho-mimicking variant Hop Y354E showed clear differences compared to the wild-type (Fig 4B and Supplementary Table S1). The SAXS data indicate that Hop Y354E adopts a more extended conformation in solution (increase of the D_{max}) accompanied by a conformational rearrangement as visible by a shift of the P(R) maxima at 25 and 50 Å, respectively. To obtain further details on the conformational changes, we recorded SAXS data for smaller Hop constructs containing the wild-type or mutated TPR2A–TPR2B domains. The mutation mimicking the phosphorylation leads to an increase of the D_{max} by 20 Å, similar to what we observed for the full-length proteins. Given that the P(R) shows a tailing-out at higher distances, the mutation seems to destabilize the rigid connection between TPR2A and TPR2B which in turn leads to a detachment and increased dynamics of the

domains. Using the ensemble optimization method (EOM) program [37], we find that TPR2A and TPR2B are no longer fixed in a rigid orientation in the Hop Y354E mutant but are adopting a predominantly dynamic conformation in which the two domains are disconnected (Fig 4C).

To determine the influence of the phospho-mimetic mutations on specific functional properties of Sti1/Hop, we first tested their effects on Hsp90. We found that all Hop/Sti1 phospho-mimics inhibit the Hsp90 ATPase activity comparable to the wild-type proteins (Fig 5A). Consistent with this, their affinity for Hsp90 also was not compromised, which is in agreement with the location of the phosphorylation sites in predominantly Hsp70-interacting regions of the co-chaperone. However, when we determined their affinities toward Hsp70, we found that for some of the phospho-mimicking mutations, the binding to Hsp70 was weaker (Fig 5C). The strongest
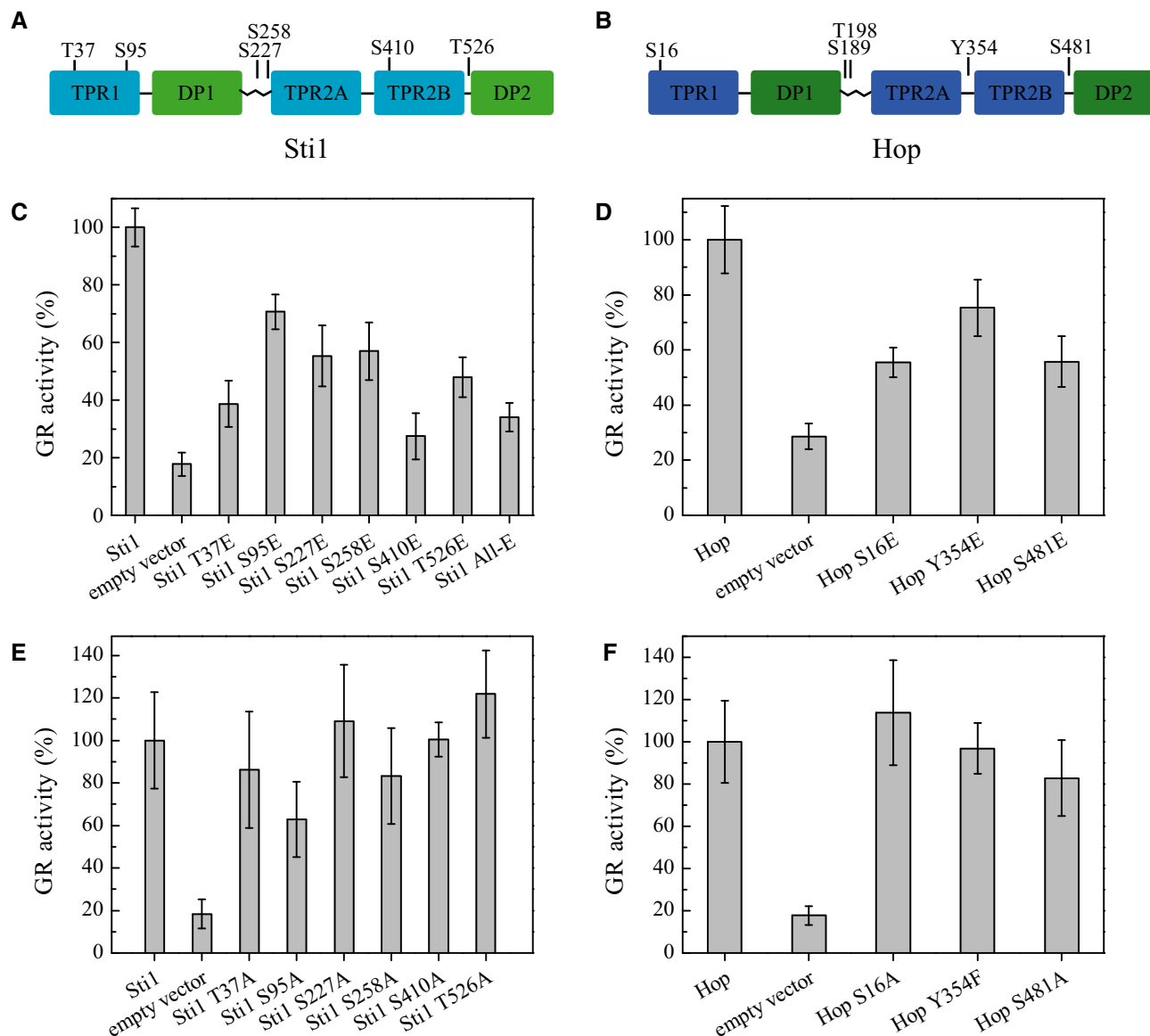
Figure 3. Phospho-mimicking variants of Sti1/Hop reduce GR activation *in vivo*.

A, B   Scheme of phosphosites in Sti1 (A) and Hop (B).

C, D   GR activation measured with a β-galactosidase-based assay in *sti1Δ* yeast cells expressing different phospho-mimicking variants of Sti1 (C) or Hop (D).

E, F   GR activation measured with a β-galactosidase-based assay in *sti1Δ* yeast cells expressing different alanine/phenylalanine variants of Sti1 (E) or Hop (F).

Data information: Means of three independent experiments are shown. Error bars indicate standard errors.

effects with an about twofold increase in the K_D value were observed for Sti1 S410E and Hop Y354E.

The tendency of the phospho-mimics to modulate binding of Hsp70 was further investigated by analytical ultracentrifugation (Fig 5B). Here, the phospho-mimics were titrated to constant amounts of labeled Hsp70. With increasing amounts of Hop/Sti1 variant added, the sedimentation coefficient of the labeled Hsp70 increased consistent with the binding of Hop/Sti1. We determined the amount of Sti1/Hop to reach complete complex formation by observing the shift in the $s_{20,w}$ value. Maximal sedimentation coefficients of the complexes formed with 4 µM of the respective phospho-mimic are depicted in Fig 5B. Interestingly, several of the

mutants showed $s_{20,w}$ values, which are lower than that of the wild-type protein under these conditions, implying that either the affinity for Sti1/Hop is reduced or the conformation of the ternary complex is altered (Supplementary Fig S3). The lowest sedimentation coefficients implying the weakest interaction were obtained with the mutants T37E, S95E, S410E, and Y354E. This analysis confirmed that the Hop/Sti1 phospho-mimetic variants are indeed compromised in their interaction with Hsp70. This allows the conclusion that phosphorylation of Sti1 and Hop at specific sites negatively affects the interaction with Hsp70. As Sti1 S410E and Hop Y354E, besides being strongest affected in Hsp70 interaction, also had an impact on GR activation, apparently these two properties of Sti1/Hop are linked.
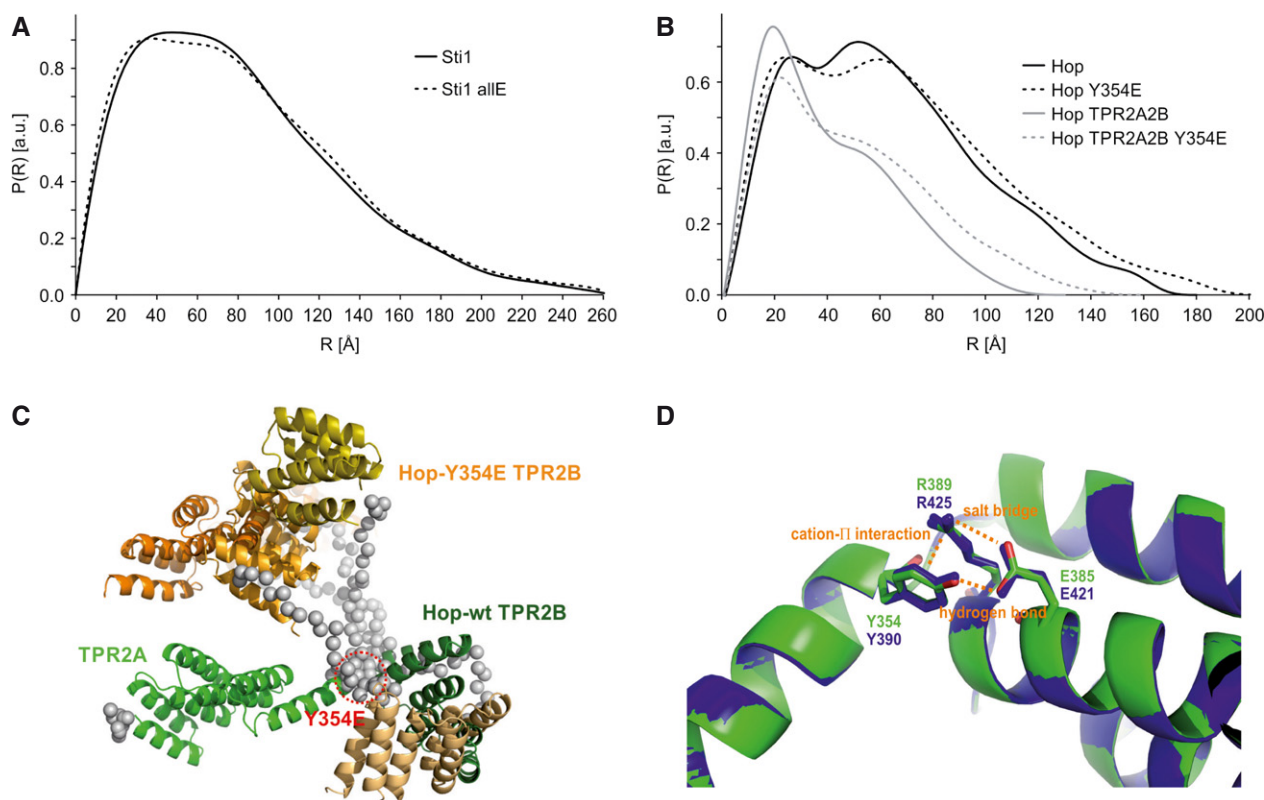
Figure 4. Impact of phospho-mimic mutations on the solution conformation of Sti1 and Hop.

A, B   SAXS data showing a comparison of the experimental radial density distributions of Sti1 (A) and of Hop constructs (B). The SAXS data of the wild-type constructs are shown as solid line, and the corresponding data obtained for the mutant samples are shown as dashed lines.

C   Comparison of the homology model of Hop TPR2A–TPR2B (green) with the model of Hop TPR2A–TPR2B Y354E generated with the ensemble optimization method (EOM) program based on SAXS data. The structures are overlaid on TPR2A (green), TPR2B of Hop is colored dark green, and the conformations of Hop Y354E TPR2B are shown in various shades of orange. The flexible linker residues are represented as Cα dummy atoms by the program CORAL and are shown as gray spheres.

D   Structural basis for TPR2A–TPR2B rigidity. Overlay of the crystal structure of Sti1 TPR2A–TPR2B (PDB-ID: 3UQ3; blue) with the homology model of Hop (green). Key residues and interactions forming the triangular interaction network are labeled. The Hop phosphorylation site Y354 is a key element for the rigid arrangement and is engaged in an interaction network involving a cation-π interaction with R389 and a hydrogen bond with E385, and a salt bridge between E385 and R389 which stabilizes the orientation between TPR2B and the α-helix connecting TPR2A and TPR2B.

Discussion

Sti1 and Hop are two highly conserved co-chaperones which act as adaptors for the Hsp90 and Hsp70 machinery [10]. They share strong sequence and structural similarity although being separated by 500 million years of evolution.

Regarding their interaction with Hsp90 and Hsp70 *in vitro* and the domain-specific functions *in vivo*, Sti1 and Hop generally behaved similarly in our assays. Thus, the basic traits of Sti1 and Hop are conserved. Differences in the inhibition of the Hsp90 ATPase activity are conveyed not by Sti1/Hop but rather by the respective Hsp90 homolog, which show striking differences between yeast and human Hsp90's enzymatic properties. The finding that human Hsp90 could not be inhibited by Sti1/Hop to the same degree as yeast Hsp90 may be related to the fact that inhibition of yHsp90 is more relevant than that of the human homolog, as the yeast Hsp90 ATPase rate is about 10-fold higher than that of human Hsp90 [38]. Thus, the human Hsp90 cycle is already substantially slower than that of yeast and a complete inhibition may not be of equal importance. Small differences though exist for the two

homologs in their affinities toward Hsp90 and Hsp70. Interestingly, in *C. elegans*, the Sti1/Hop homolog is considerably shorter and contains only the core part of the protein TPR2A–TPR2B–DP2 [39]. The *C. elegans* homolog fulfills the essential functions of Hop, although Hsp90 decreases the affinity for Hsc70 considerably [39].

Sti1/Hop only indirectly recruits clients via the interaction with client-bound Hsp70. Therefore, it is strongly correlated in its evolution to Hsp90 and Hsp70 which themselves are conserved in their basic architecture. Other client-recruiting co-chaperones like Cdc37 act via direct interaction with a particular subset of clients [5,7]. In this context, for the kinase-specific co-chaperone Cdc37 sequence, conservation is low and binding sites in Hsp90 as well as its regulatory impact on Hsp90 are different in divergent eukaryotes [40–42].

In the case of Sti1/Hop, specific adaptations seem to be mediated via post-translational modifications. The major functional differences between Sti1 and Hop observed in this study are conveyed by phosphorylation which occurs almost exclusively at unique sites. Typical for active regulatory phosphorylation sites, they are mostly found in loops or unfolded regions [43]. However, while the specific
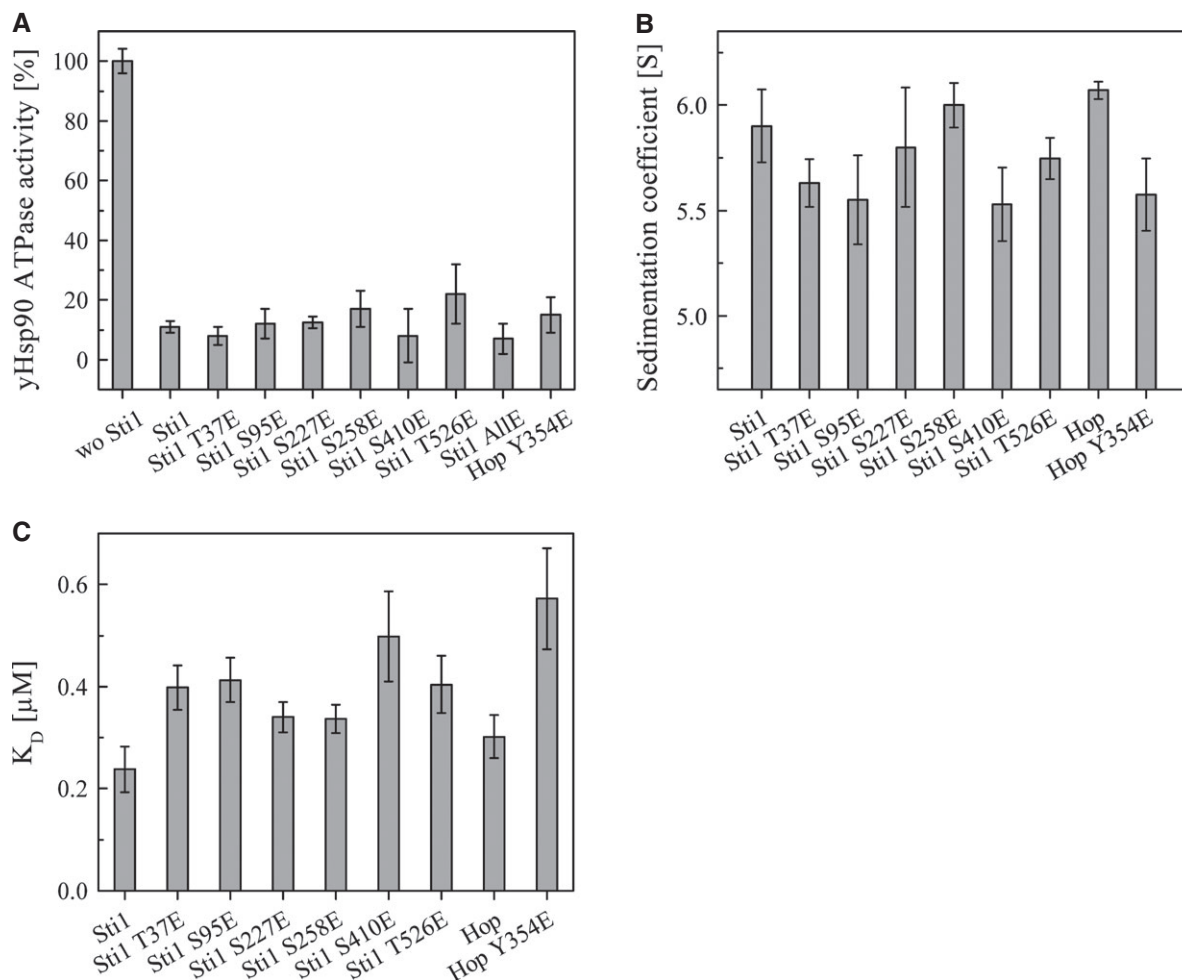
Figure 5. Interaction of Sti1/Hop phospho-mimics with Hsp90 and Hsp70.

A ATPase activity of yHsp90 upon addition of Sti1/Hop phospho-mimicking mutants measured with an ATP-regenerative ATPase assay. Of 2 μM yHsp90 (30°C) was used with equimolar concentrations of Sti1/Hop variants. Means of three independent experiments are shown with the standard error.

B Comparison of sedimentation coefficients of Hsp70-Sti1/Hop complexes using 500 nM labeled yHsp70 and 4 μM of Sti1/Hop phospho-mimic. Means of three independent experiments are shown with the standard error.

C Affinities of Sti1/Hop phospho-mimics with yHsp70 as quantified by SPR. Error bars indicate standard error of the fit using titration data.

sites are unique, a pattern exists. In both Sti1 and Hop, phosphorylation occurs in the TPR1 domain, at sites in the long linker region and in the connection between TPR2B and DP2, while only rarely Hsp90-interacting domains are targeted. The phosphorylation site in the linker between DP1 and TPR2A is conserved from yeast to men and may be important for the potential interplay between TPR2B and DP2.

The site targeting the linker between the TPR2A and TPR2B domains is unique for several reasons. Here, phosphorylation affects a conserved tyrosine, but only mammalian Hop is phosphorylated. Although tyrosine phosphorylation is possible in yeast, our results indicate that the respective tyrosine residue in mammalian Hop is not modified in yeast [44]. It influences a region which is very important for the orientation of the two domains in this central element of Sti1/Hop [21]. Mutations in this segment also lead to a loss of function [21]. This site is of special interest, as the respective

phospho-mimetic mutant is the only one for which larger structural changes could be detected compared to the wild-type protein. SAXS experiments suggest that this mutation leads to a disruption of the rigid interaction of the two TPR domains. The different conformations of wild-type and mutated Hop can be explained structurally. Y354 is engaged in a triangular interaction network involving a cation-π interaction with R389, a hydrogen bond with E385, and a salt bridge between E385–R389, thereby fixing the orientation between TPR2B and the α-helix connecting TPR2A and TPR2B (Fig 4D). In the Y354E mutant, the cation-π interaction is absent which in turn leads to a destabilization of the TPR2A–TPR2B arrangement. This leads to a twofold decrease in the affinity for Hsp70 and decrement of the *in vivo* function of Hop concerning GR activation.

For the modifications in the linker regions, both the phospho-mimetic mutation of S227 and S258 in Sti1 consistently resulted in a

50% reduction in GR activity. Furthermore, both mutations led to an identical slight increase in the K_D for Hsp70. Similarly, the modification in the TPR1 domain of Sti1 or Hop (at different positions) decreased GR activity and slightly reduced the affinity for Hsp70. For Sti1, the strongest effect is seen for S410, which shows the most pronounced reduction in GR activity and the largest increase in the K_D for binding Hsp70. Interestingly, this site is in TPR2B, one of the two Hsp70-interacting domains of Sti1. A homologous phosphorylation site has not been found in Hop yet.

It is suggestive to assume that the changes in affinity for Hsp70 are the reason for the decreased GR activity. In general, one can conclude that phosphorylation occurs at hotspots near the Hsp70 binding region and the linker connecting the two Hsp70 binding sites. In line with this, the phospho-mimetic mutations reduced the affinity for Hsp70 while not affecting the interaction with Hsp90. Interestingly, the affinity for Hsp70 is affected by modifications of different segments of Sti1. This argues for the physiological importance of both putative Hsp70 binding sites in Sti1. Why Hsp70 needs to interact with two sites in Sti1 is still enigmatic. This switch of binding sites may be related to the handing-over of clients to Hsp90, and in this context, phosphorylation may decrease the transfer efficiency of clients.

Hop seems to be more tightly regulated by phosphorylation compared to Sti1 as phosphorylation influences Hop not only concerning the interaction with Hsp70. Two phosphorylation sites in Hop (S189, T198) are involved in the translocation to the nucleus [35,36]. In addition, a phospho-mimicking mutant of T198 negatively affected the interaction with Hsp90 [36].

In recent years, evidence has accumulated that regulation by phosphorylation plays a tremendous role for the Hsp90 machinery in general. Hsp90 itself is heavily phosphorylated. Phosphorylation of yeast Hsp90 leads to a decrease in its activity [45]. Interestingly, the phosphorylation sites are also well distributed over the entire molecule, and for human Hsp90, tyrosine phosphorylation has been shown to play an important role [45–47], like defined here for Hop. In the case of Sti1/Hop, phosphorylation adds another layer of regulation in addition to its induction by heat shock [11]. A positive effect of phosphorylation has been demonstrated for Cdc37. In this case, phosphorylation at a specific serine is required for chaperoning kinases. The finding that both Sti1 and Hop are phosphorylated at multiple sites supports the importance of this co-chaperone for the Hsp90 machinery. Although the deletion of Sti1 in yeast is not lethal [11], a tight regulation seems to be required, especially in the case of Hop. This may allow directing the Hsp90 machinery to specific functions and a subset of its clientele.

Materials and Methods

Protein purification

For Sti1/Hop variants, yHsp82 and hHsp90β, pET28 vectors carrying the respective genes plus an N-terminal 6xHis-SUMO-tag or a thrombin-cleavable 6xHis-tag were transformed into the *E. coli* strain BL21 (DE3) Codon Plus. For Ssa1, the *Pichia pastoris* strain KM71H-Ssa1 (aox1::ARG4; arg4; 6xHis-SSA1 gene genomically inserted at AOX1 locus) was used. Proteins were purified as described in [21] point mutations of Sti1 and Hop were generated using the Quik-Change II Site-Directed Mutagenesis Kit (Agilent, La Jolla, USA).

ATPase assay

ATPase activities were measured using a regenerating ATPase assay as described before [21]. Assays were performed in 50 mM Hepes (pH 7.5), 50 mM KCl, 5 mM MgCl$_2$, and 2 mM ATP. ATPase activity was measured at 30°C using 2–4 μM yHsp90 or at 37°C with 5–10 μM hHsp90.

Analytical ultracentrifugation

Analytical ultracentrifugation was performed in a Beckman ProteomeLab XL-A (Beckman, Fullerton, USA) equipped with a fluorescence detection system (Aviv Biomedical, Lakewood, USA) using 5-(and-6)-carboxyfluorescein coupled Ssa1 or hHsp70. Sedimentation analysis was carried out at 42,000 rpm in a TI-50 Beckman rotor (Beckman, Fullerton, USA) at 20 °C in 10 mM potassium phosphate (pH 7.5) as described in [21].

GR activity assay in yeast cells

The *sti1Δ* yeast strain YOR027w (BY4741; *Mat a*; *his3Δ1*; *leu2Δ0*; *met15Δ0*; *ura3Δ0*; YOR027w::kanMX4, from Euroscarf) was transformed with the human glucocorticoid receptor (hGR) expression vector (p413GPD-hGR), the reporter plasmid with GR response elements pUCΔSS-26X [48] and a p425GPD expression plasmid for Sti1 and Hop variants. Single clones were grown at 30°C in minimal medium to stationary phase. The β-galactosidase assay was performed as described in [21]. The relative activity values were obtained by setting the absolute value of the full-length signal to 100%. Results are the mean of three independent experiments. Error bars indicate standard error.

SPR

SPR measurements were carried out with a BiacoreXTM instrument (GE Healthcare, München, Germany). Hsp70 or Hsp90 were covalently linked to a CM5 SPR chip. The analyte was flushed over the chip in 40 mM Hepes, 20 mM KCl, 5 MgCl$_2$, 0.005% Tween (v/v) at 20°C. The SPR signal change upon injection was subtracted for the reference signal and plotted against the corresponding concentration.

SAXS

All SAXS data for the Sti1 and Hop constructs were recorded, processed, and analyzed as described in the Supplementary Methods.

Author contributions

AR, EB, FT, and BS carried out the biochemical analyses and the *in vivo* studies. KR collected the analytical ultracentrifugation data. TM performed the SAXS experiments. AR, TM,KR, and JB wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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