Hsp90 regulates the dynamics of its cochaperone Sti1 and the transfer of Hsp70 between modules

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The cochaperone Sti1/Hop physically links Hsp70 and Hsp90. The protein exhibits one binding site for Hsp90 (TPR2A) and two binding sites for Hsp70 (TPR1 and TPR2B). How these sites are used remained enigmatic. Here we show that Sti1 is a dynamic, elongated protein that consists of a flexible N-terminal module, a long linker and a rigid C-terminal module. Binding of Hsp90 and Hsp70 regulates the Sti1 conformation with Hsp90 binding determining with which site Hsp70 interacts. Without Hsp90, Sti1 is more compact and TPR2B is the high-affinity interaction site for Hsp70. In the presence of Hsp90, Hsp70 shifts its preference. The linker connecting the two modules is crucial for the interaction with Hsp70 and for client activation in vivo. Our results suggest that the interaction of Hsp70 with Sti1 is tightly regulated by Hsp90 to assure transfer of Hsp70 between the modules, as a prerequisite for the efficient client handover.
Hsp90 and Hsp70 are ubiquitous molecular chaperones. While Hsp70 associates with newly synthesized peptide chains, Hsp90 is required during later stages of folding and serves a more specialized set of client proteins. Many of them are involved in signalling pathways, such as kinases and transcription factors. For eukaryotic Hsp90, several cochaperones exist that stabilize specific conformations of Hsp90 and Hsp70 that interacts with the TPR (tetratricopeptide repeat) domains of some cochaperones. The most common interaction site is the C-terminal EEVD motif of Hsp90 and Hsp70 that allows client activation. The differences in the individual domains are known but their overall arrangement remains enigmatic. The TPR domains are tandem repeats of a 34-amino-acid consensus motif, which forms a cleft of seven antiparallel alpha-helices capable of binding the C-terminal tails of Hsp90 and Hsp70. In addition, substantial contacts are formed between the Hsp90 M-domain and TPR2B. Together, TPR2A–TPR2B stabilizes the open conformation of Hsp90 and inhibits its ATPase activity.

Simultaneous to Hsp90 binding, Sti1 is able to interact via TPR1 and TPR2B with Hsp70 (refs 22,28,29). However, it was unclear whether two Hsp70 molecules can contact TPR1 and TPR2B at the same time. Strikingly, deletion of TPR1 does not affect the ability of Sti1 to activate clients in vivo. Therefore, the physiological relevance of the Hsp70-binding function of TPR1 remained unclear. Another intriguing feature of Sti1 is that each of the two Hsp70-binding TPR domains is followed by a DP (aspartate and proline rich) domain (Fig. 1a). The structures of the individual domains are known but their overall arrangement remains enigmatic. The TPR domains are tandem repeats of a 34-amino-acid consensus motif, which forms a cleft of seven antiparallel alpha-helices capable of binding the C-terminal tails of Hsp90 and Hsp70 (ref. 23). In Sti1, the peptide-binding groove of TPR2A is highly specific for Hsp90. In addition, substantial contacts are formed between the Hsp90 M-domain and TPR2B. Together, TPR2A–TPR2B stabilizes the open conformation of Hsp90 and inhibits its ATPase activity.

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Interestingly, although the two DP domains of Sti1 are structurally similar, DP2 cannot be exchanged by DP1 in terms of biological function.

Figure 1 | Overall shape of Sti1 and Sti1 fragments. (a) Scheme of Sti1 and Sti1 deletion variants investigated in this study. (b) Molecular mass of Sti1 variants as calculated assuming globular proteins (grey) and experimentally derived from analytical gel filtration experiments (black). The differences between these indicate that Sti1 fragments containing the linker exhibit an elevated hydrodynamic radius and possess an elongated shape in solution. (c,d) $D_{max}$ (left) and $R_g$ (right) distributions of the ensemble optimization method models of wild-type Sti1 and mutants in the initial pool of structures with randomized interdomain linkers (dashed lines) and in the selected ensembles (solid lines).
To resolve the molecular mechanism of Sti1, we analysed the structural dynamics of the modules in Sti1 and their interaction with Hsp70 and Hsp90. We show that Sti1 contains two functional modules separated by a linker. Binding of Hsp90 affects the interaction of Hsp70 with the two potential binding sites in Sti1.

Results

Sti1 is an elongated protein with a flexible hinge region. To obtain insight into the arrangement of the domains in full-length Sti1, we performed analytical gel filtration experiments and single-pair Förster Resonance Energy Transfer (spFRET) experiments with wild-type Sti1 and various mutants. The molecular mass for the wild-type protein determined using analytical gel filtration was largely increased compared with the calculated values for globular proteins (Fig. 1a,b). As previous studies showed that Sti1/Hop is a monomer\(^3\), this indicates an elongated shape of Sti1. To confirm this, we performed analytical ultracentrifugation experiments with Sti1 and a Sti1 variant, lacking the linker region. The sedimentation data indicate that both proteins are monomeric. We obtained frictional coefficients of 1.80 ± 0.05 and 1.76 ± 0.06, respectively, suggesting that both are elongated. Solution-based spFRET experiments on constructs where the donor and acceptor molecules were fluorescently labelled near the C- and N-terminus of Sti1 (S2C-G588C, S2C-S523C or G131C-S523C) exhibited no appreciable FRET signal (Supplementary Fig. 1). This indicates that the separation of fluorophores is larger than ~100 Å. These results are in agreement with an elongated shape of Sti1 as previously suggested\(^3\). Furthermore, it may also be an indication of flexibility between modules. The unusual diffusion behaviour was also observed for the constructs that contained the linker region connecting DP1 and TPR2A (ADP2, TPR1–DP1–TPR2A and DP1 + linker). In all other cases (TPR1–DP1, TPR2A–TPR2B, TPR1, TPR2A–TPR2B–DP2, TPR2B–DP2, TPR2B and DP2), the calculated and experimental molecular weights correlated (Fig. 1b). Together, this indicates that the linker is responsible for the elongated and possible dynamic nature of Sti1. To further address the conformation of Sti1, we performed small-angle X-ray scattering (SAXS) analysis. Scattering curves recorded at severalolute concentrations confirmed that Sti1 constructs containing the linker region adopt an extended conformation as we observed a high radius of gyration and a large maximum diameter (for Sti1 wild-type: \(R_g = 58\) Å and \(D_{max} = 260\) Å, Supplementary Fig. 2a,d). We analysed the conformational space sampled by Sti1 and fragments thereof using the ensemble optimization method (EOM)\(^3\). Here, a pool of independent structures based on the amino-acid sequence and available structural information is first generated. A genetic algorithm is then used to select an ensemble of structures that best describes the experimental SAXS data (for more details see the Methods section). We identified the best ensembles concerning the agreement of experimental and back-calculated data (Supplementary Fig. 2c,e, see Methods) and compared the \(R_g\) and \(D_{max}\) distributions of the random pool of structures with the selected ensembles (Fig. 1c,d). For full-length Sti1, the selected ensemble clearly shows a higher degree of compaction compared with the random pool (Fig. 1c). This suggests that there must be inter-domain interactions within Sti1 that lead to a partial compaction of the molecule. On the basis of the \(R_g\) and \(D_{max}\) distributions of Sti1, TPR2A–TPR2B–DP2, ADP2, \(\Delta\)linker, TPR1–DP1 and TPR2B–DP2 (Fig. 1c,d; see also Table 1 and Supplementary Fig. 2c for the structural ensembles), we find that the compaction is strongest within the TPR2B–DP2 module. The maxima of \(R_g\) and \(D_{max}\) distributions of constructs lacking the TPR2B–DP2 module (for example, ADP2, TPR1–DP1) is close to the maxima found for a random pool, and therefore compact conformations must be underestimated in these ensembles. Deletion of the linker connecting DP1 and TPR2A (\(\Delta\)linker) resulted in a substantial reduction of the molecular dimensions (\(R_g = 58.4\) Å versus 50.1 Å; \(D_{max} = 264\) Å versus 207 Å). In summary, these results show that the segment connecting TPR1 and DP1, as well as the linker between DP1 and TPR2A, is flexible and within these regions the Sti1 domains behave like beads on a string. In contrast, \(R_g\) and \(D_{max}\) distributions of constructs including the TPR2B–DP2 module (for example, \(\Delta\)TPR1, TPR2A–TPR2B–DP2, \(\Delta\)linker, TPR2B–DP2) show a shift towards lower \(R_g\) and \(D_{max}\) indicative of the presence of more compact conformations. In concordance with this, the \(D_{max}\) of TPR2A–TPR2B–DP2 and TPR2B–TPR2B is identical (both 130 Å; Supplementary Fig. 2a), indicating that the presence of the DP2 domain does not lead to an extension of the maximum diameter.

**Table 1 | SAXS data and analysis.**

| Sample             | Stoichiometry | \(R_g\) (Å) | \(D_{max}\) (Å) | Molecular mass (kDa) |
|--------------------|--------------|------------|----------------|---------------------|
| Sti1               |              | 58.4 ± 0.6 | 264 ± 70       | 70                  |
| Sti1 \(\Delta\)linker |              | 50.1 ± 0.5 | 207 ± 60       | 60                  |
| Sti1–\(\Delta\)TPR1|              | 54.0 ± 0.1 | 200 ± 48       | 48                  |
| Sti1–TPR2A–TPR2B–DP2|             | 36.0 ± 0.1 | 130 ± 35       |                     |
| Sti1–ADP2          |              | 60.3 ± 0.1 | 220 ± 58       |                     |
| Sti1–TPR1–DP1      |              | 30.4 ± 0.1 | 100 ± 29       |                     |
| Sti1–TPR2B–DP2     |              | 23.2 ± 0.1 | 80 ± 17        |                     |
| Sti1–TPR2A–TPR2B   |              | 33.9 ± 0.1 | 130 ± 24       |                     |
| Hsp70              |              | 40.0 ± 0.1 | 140 ± 70       |                     |
| Sti1 + Hsp70       | 1:1          | 71.2 ± 0.1 | 240 ± 135      |                     |
|                   | 1:2          | 76.2 ± 0.1 | 280 ± 123      |                     |
| Sti1–\(\Delta\)linker + Hsp70 | 1:1 | 60.1 ± 0.1 | 200 ± 130      |                     |
|                   | 1:2          | 59.7 ± 0.1 | 200 ± 100      |                     |
| Sti1–TPR1 + Hsp70  | 1:1          | 45.0 ± 0.2 | 160 ± 86       |                     |
|                   | 1:2          | 45.7 ± 0.1 | 170 ± 80       |                     |

*The molecular mass was determined from the scattering intensity at zero angle (\(\theta/2\)) using ISA as a reference.

TPR2A, TPR2B and DP2 of Sti1 form a rigid module. As the SAXS data suggested that in the Sti1 TPR2A–TPR2B–DP2 segment DP2 is in contact with TPR2B, we compared NMR spectra of \(^{15}\)N-labelled TPR2B-DP2 with the spectra of the isolated domains (Fig. 2a). Shifts appeared not only for residues in the region connecting the two domains (residues S19 to S525) but also for residues in the DP2 domain (Fig. 2a). These are located in the first, second and the last helix of DP2 and also in the C-terminal end of TPR2B indicating an interaction between the two domains. We also performed paramagnetic relaxation enhancement (PRE) NMR measurements on TPR2B–DP2 constructs where cysteines were introduced at various positions within the DP2 domain (E525C, K536C, Q545C, N559C and N589C) and modified with a PROXYL spin label (Fig. 2b and Supplementary Fig. 3). Relaxation enhancement was mainly observed in DP2 but also residues in TPR2B and the linker connecting the two domains were affected. The strongest effects on TPR2B were observed when the spin label was attached to E525C (in the linker between TPR2B and DP2). Moreover, when the spin label was attached to a residue in the second helix of DP2 (K536C and Q545C), PRE effects were visible in the C-terminal helix of TPR2B and the linker region. Together with the chemical shift analysis data,
these observations show that interdomain contacts are formed between the C-terminal helix of TPR2B, the linker and helix 1 and 2 of DP2.

To gain detailed insight into the organization of Sti1, we used spFRET as this method not only allows determination of distances on the molecular scale, but can also be used to investigate the flexibility of biomolecules. To look for dynamics within TPR2A–TPR2B–DP2 module, cysteines were introduced at positions G309C (TPR2A) and S523C (linker between TPR2B and DP2) (Supplementary Fig. 4a). The spFRET histogram has a single dominant peak with an FRET efficiency of $\approx 20\%$ and a donor–acceptor separation of $\approx 65\ Å$ (Supplementary Fig. 4b). A photon distribution analysis of the spFRET data indicates that the main FRET peak can be described by a single population with a Gaussian distribution exhibiting a s.d. of $5\ Å$. This indicates some flexibility between the FRET pair, but, considering that different domains were fluorescently labelled, this width is surprisingly narrow. In addition, no dynamic fluctuations were observed in the spFRET measurements on the TPR2A–TPR2B–DP2 module (Supplementary Fig. 4c). The idea of a rigidly connected TPR2B–DP2 segment derived from the spFRET experiments is in excellent agreement with the structural data, which also suggest that the orientation of DP2 with respect to TPR2B is fixed. In our structural model, DP2 is in contact with the last three $\alpha$-helices of TPR2B and does not interfere with the Hsp70/Hsp90 peptide-binding site (Fig. 2c). In summary, our analysis shows that Sti1 is an elongated molecule in which a rigid C-terminal module consisting of three domains (TPR2A–TPR2B–DP2) cooperates with a two-domain N-terminal module (TPR1–DP1).
Sti1 has two differentially activated Hsp70-binding sites. As demonstrated previously, Hsp70 can bind alternatively to TPR1 and TPR2B. This is consistent with our SAXS analysis where Hsp70 is shown to bind TPR1 and TPR2B individually (Supplementary Fig. 2b,f). Our SAXS experiments also show that the molecular weight of the main complex between full-length Sti1 and Hsp70 does not change comparing a 1:1 to a 1:2 stoichiometry (Fig. 3a). As we used concentrations above both individual binding affinities, we conclude that full-length Sti1, although containing two Hsp70-binding sites, binds to Hsp70 in a 1:1 stoichiometry (Fig. 3a). This is in accordance with previous findings on the stoichiometry of the interaction of Hop and Hsp70 (refs 35,36).

We further assessed the interaction of Hsp70 with Sti1 via surface plasmon resonance spectroscopy (SPR) analysis using an Hsp70-coupled chip (Fig. 3b). Both isolated TPR1 and TPR2B constructs specifically bound Hsp70 but exhibited weaker affinity towards Hsp70 compared with wild-type Sti1 (three- to fivefold), suggesting that additional interactions take place in the full-length construct. To better understand the role of the different domains for binding of Hsp70, we investigated full-length Sti1 constructs with specific mutations in the peptide-binding groove of the two TPR domains (mutation in TPR1: N39A, mutation in TPR2B: R469A) to disrupt the interaction with Hsp70 (Fig. 3b). These mutations were designed based on structural information on the interaction of the C-terminal peptide of Hsp70 and the respective TPR domain. Surprisingly, the introduction of a TPR1-inactivating mutation (N39A) did not affect the affinity of Sti1 towards Hsp70. Likewise, fragments of Sti1 lacking TPR1 (TPR2A–TPR2B and TPR2A–TPR2B–DP2) exhibited similar dissociation constants. In contrast, mutation of the peptide binding groove of TPR2B (R469A) or complete loss of TPR2B.
(TPR1–DP1–TPR2A) reduced the affinity towards Hsp70 compared with wild-type Sti1. These results are consistent with both TPR1 and TPR2B being capable of binding Hsp70 in Sti1. However, the high-affinity interaction is mediated by the TPR2B domain and an intact TPR2A–TPR2B segment is necessary for the wild-type-like interaction with Hsp70.

To gain insight into the formation of ternary complexes, we employed analytical ultracentrifugation (AUC) (Fig. 3c–e). Sti1 variants were titrated to constant amounts of Hsp90 and fluorescently labelled yHsp70 (Hsp70*, Ssa1). In these experiments, generally three Hsp70-containing species can be observed: Hsp70* alone (4.2 S, left line), binary complexes of Hsp70* with Sti1 (5.6 S, middle line) and ternary complexes of Hsp70* with Sti1 and Hsp90 (8–10 S depending on saturation, right line). The first two complexes cannot be separated completely as the peaks in dc/dt plots overlap strongly. After addition of Sti1 to *Hsp70 and Hsp90, ternary complexes are formed at higher sedimentation coefficients (Fig. 3c), while in the absence of Sti1 *Hsp70 sediments as a single species with 4.2 S. At Sti1 concentrations between 0.5 and 4 μM more and more of the ternary complexes are formed, leaving only little amounts of free *Hsp70 observable at 4.2 S. With the TPR1-defective (Fig. 3d) as well as with the TPR1-lacking construct (Fig. 3e), ternary complexes were formed indicating that simultaneous binding of Hsp90 and Hsp70 occurs via binding to TPR2A–TPR2B. However, for these variants, ternary complex formation was weakened compared with wild-type Sti1. Taken together, these results emphasize that, while both TPR1 and TPR2B contribute to the interaction with Hsp70, TPR1 becomes more important as an Hsp70-binding site when Hsp90 is present. Furthermore, the data suggest that TPR1 and TPR2B are not independent in their interaction with Hsp70 but that there is communication between these domains in the context of Sti1.

The Sti1 linker affects client activation in vivo. The two modules of Sti1 are connected to each other via a flexible linker. To investigate the biological function of this linker, we replaced it by a short Ser-Ala-Gly-Ala segment (∆linker, Fig. 1a) and tested the effects of the linker deletion on the activation of the glucocorticoid receptor (GR), a stringent Hsp90 client, in vivo. A S. cerevisiae sti1 knockout was transformed with plasmids for Sti1 variants, the GR and a β-galactosidase-based reporter system. In the absence of Sti1, GR activity was reduced to 20%. When the linker deletion mutant was expressed, the levels of activated GR increased as long as Sti1 concentrations were below 3 μM, apparently not as much Hsp90 was incorporated into these complexes. Hence, in vitro, the population of binary and ternary complexes depends strongly on the ratio of the three components. Importantly, for the Sti1 linker, the ternary complex was never more abundant than the binary complex, which would indicate a weaker affinity for Hsp90.

In addition to the linker deletion, we introduced a mutation in TPR1 (N39A) and tested for ternary complex formation. Surprisingly, we detected increased ternary complex formation for this construct as compared with wild-type Sti1, even though the linker-deletion and N39A alone had the opposite effect (Fig. 5c). Together with the finding that a peptide-binding defective mutation in TPR2B enhances ternary complex formation, this indicates that both Hsp70-binding sites contribute differently to ternary complex formation and that the effect of the linker on the formation of ternary complexes is strongly influenced by the interaction of Hsp70 with TPR1.

The linker is important for Hsp70 interaction. To understand the role of the linker, we performed further in vitro analyses with the linker deletion. The stability and secondary structure of this mutant were comparable to wild-type Sti1 consistent with its unstructured character. In ATPase activity assays, the Sti1 ∆linker construct inhibited the ATPase of Hsp90 as efficient as wild-type Sti1 or the TPR2A–TPR2B fragment (Fig. 4b). Moreover, as shown by SPR analysis, the deletion of the linker did not affect the affinity of Sti1 towards Hsp90 (Fig. 4c). Hence, the linker deletion does not affect the Hsp90–Sti1 interaction.

Next, we assessed the interaction of the linker-deletion construct with Hsp70. SAXS experiments showed that the linker-deletion construct binds Hsp70 in a 1:1 stoichiometry (Fig. 4d) like wild-type Sti1. In AUC experiments, the Sti1 variants were titrated to constant amounts of labelled Hsp70. Complex formation with Sti1 could be observed by the peak shift to a maximum of about 5.6 S compared with 4 S for unbound Hsp70* (Supplementary Fig. 5). Surprisingly, the maximum sedimentation coefficient was already reached at lower concentrations of the linker-deleted variant compared with wild-type Sti1, indicating a tighter interaction with Hsp70 (Fig. 4e). SPR confirmed that the linker deletion increased the affinity for Hsp70 threelfold (Fig. 4c). In the Sti1 ∆linker N39A construct where the binding of Hsp70 to TPR1 is impaired, no difference in binding affinity was observed compared with the Sti1 ∆linker variant without the point mutation (Fig. 4c). Taken together, our findings suggest that conformations enabled by the linker interfere with the binding of Hsp70 to TPR2A–TPR2B in wild-type Sti1.

The linker affects ternary complex formation. To determine how the linker deletion affects the formation of ternary Hsp90–Sti1–Hsp70 complexes, we again performed AUC experiments using constant amounts of *Hsp70 and titrating in wild-type Sti1 or the linker-deleted Sti1 variant in the presence of unlabelled Hsp90 (Fig. 5a,b). Like before (see Fig. 3c), we observed more ternary Hsp90–Sti1–Hsp70 complexes (9.5 S) with increasing amounts of Sti1. At Sti1 concentrations higher than 3 μM, ternary complexes are reduced at the expense of *Hsp70-Sti1 complexes observable at 5.6 S. This suggests that there is now sufficient Sti1 to form individual binary complexes with *Hsp70 and Hsp90, thereby disrupting partly the ternary complexes. For the linker-deletion experiments, the formation of ternary complexes is reduced at the expense of *Hsp70-Sti1 binary complexes, which form at 5.6 S. While here also the amount of ternary complexes increased as long as Sti1 concentrations were below 3 μM, apparently not as much Hsp90 was incorporated into these complexes. Hence, in vitro, the population of binary and ternary complexes depends strongly on the ratio of the three components. Importantly, for the Sti1 ∆linker, the ternary complex was never as abundant and the binary complex was always more populated. This indicates that the deletion of the linker shifts the equilibrium of complexes towards higher amounts of binary complexes and a lower population of the ternary complex, which would indicate a weaker affinity for Hsp90.
of the spFRET distribution were observed on the binding of Hsp70 and/or Hsp90 (Fig. 6b,c). Interestingly, there is an increase in the width of the spFRET distribution on binding of Hsp70, or Hsp70 and Hsp90, which indicates that chaperone binding increases the flexibility of Sti1. For the other mutants, a significant conformational change in Sti1 to lower FRET values was observed in the presence of Hsp90, indicating an increase in the distance between the two dyes (Fig. 6c and Supplementary Fig. 1).

A detailed analysis of the G193C–G309C construct revealed the presence of at least three different conformations (Fig. 6c,d). In one conformation, the distance between the labels on the DP1 and TPR2A domains is extended and a low-FRET signal is detected (low-FRET conformation with a FRET efficiency of 15%). A second high-FRET conformation ($E = 95\%$) is also observable where the two domains are close to each other. A third intermediate-FRET conformation can be seen at $E \approx 50\%$, particularly in the presence of Hsp70 or Hsp90 or both Hsps. Together this suggests that different conformations of Sti1 exist and that Hsp90 and Hsp70 modulate the equilibrium between these conformations.

SpFRET experiments visualize dynamics of the Sti1 complex. A plot of the FRET efficiency determined using fluorescence intensity versus the fluorescence lifetime of the donor reveals the dynamics between the different conformations (Fig. 6d)\textsuperscript{39}. For spFRET measurements with multiple static populations, the populations should follow the static line (shown in red in Fig. 6d). For Sti1 in the absence of Hsps, the distribution of FRET efficiency versus donor lifetime is symmetrically distributed around the static curve. The data are not scattered about the static line but deviates to the right for measurements performed with Hsp70 and/or Hsp90, suggesting the presence of dynamic fluctuations between the different conformations (dashed green line).

![Figure 4](image-url)
Figure 5 | Deletion of the linker alters ternary complex formation. (a,b) Formation of ternary complexes between γHsp70, γHsp90 and either (a) wild-type Sti1 or (b) Sti1 Δlinker was visualized by analytical ultracentrifugation. The 0.5 μM fluorescein-labelled γHsp70 and 3 μM γHsp90 were incubated with 0.5 (black), 1 (navy), 2 (blue), 3 (cyan), 4 (wine), 6 (pink) and 8 μM Sti1 (light magenta) variant in 10 mM potassium phosphate at pH 7.5. Centrifugation was performed at 20 °C and 42,000 r.p.m. Sedimentation profiles were converted into dc/dt plots according to the standard procedures and fitted with bi-Gaussian functions. For clarity, only fits are shown. The s.e. values were below 1%. (c) Formation of ternary complexes between γHsp70, γHsp90 and wild-type Sti1 (black), Sti1 Δlinker N39A (red), Sti1 N39A (blue) and Sti1 Δlinker (pink) visualized by analytical ultracentrifugation. The 0.5 μM fluorescein-labelled γHsp70 and 3 μM γHsp90 were incubated with 3 μM Sti1 variant in 10 mM potassium phosphate at pH 7.5. Centrifugation was performed at 20 °C and 42,000 r.p.m. Sedimentation profiles were converted into dc/dt plots according to the standard procedures and fitted with bi-Gaussian functions. For clarity, only fits are shown. The s.e. values were below 1%.

lines in Fig. 6d). Fluctuations between different conformations on the millisecond to second timescale can be directly measured using spFRET on immobilized samples providing complementary information to the FRET histograms discussed above. Here, we encapsulated single Sti1 molecules with or without Hsps in vesicles, which were immobilized on the surface of quartz prisms (see Materials and Methods). To verify that encapsulation had no deleterious effects on the sample, burst analysis experiments of Sti1 in vesicles were compared with measurements of Sti1 without encapsulation and no significant differences were observed. Figure 6e shows exemplary surface-based spFRET traces for the G193C–G309C Sti1 construct alone and in the presence of Hsp70 and Hsp90 (also see Supplementary Fig. 6). In all cases, a fraction of molecules exhibited dynamics. For Sti1 alone, only a low percentage of traces showed dynamics (11%, Table 2). Upon the addition of Hsp70 or Hsp90, the percentage of dynamic molecules increased from 11% up to ~25%. When both Hsps were present, we observed Sti1 switching between different conformations in up to ~30% of the detected molecules. This suggests that binding of Hsp70 and/or Hsp90 to Sti1 induces dynamics in the protein that brings the TPR1 and TPR2 modules together.

To support this hypothesis, we performed surface-based spFRET experiments on Sti1 constructs with Hsp70-binding mutations in TPR1 (N39A) and TPR2B (N435A). For Sti1 alone or for Sti1 in the presence of Hsp90, the two Hsp70-binding mutants showed the same percentage of dynamics compared with Sti1 G193C–G309C (Table 2). Also for the G193C–G309C N39A construct, a similar increase in fraction of molecules exhibiting dynamics was observed in the presence of Hsp70 as with Hsp90 or with both chaperones. In contrast, the fraction of molecules showing dynamics for the G193C–G309C N435A mutant did not significantly change compared with Sti1 alone on addition of Hsp70 (15 versus 17%, respectively). Hence, binding of either chaperone to the TPR2A–TPR2B–DP2 module is sufficient to induce dynamics, but not binding of Hsp70 to TPR1.

Interestingly, when both Hsp70 and Hsp90 are bound, the two TPR mutants exhibited an increase in the fraction of dynamic molecules but this fraction was significantly less than that for wild-type Sti1. This again suggests that, for the ternary complex, both Hsp70-binding sites are important.

Kinetic information was extracted from the spFRET traces using a Hidden Markov model (HMM) analysis (Tables 3 and 4). Three FRET states were detected with FRET efficiencies of ~20% (low FRET), ~60% (intermediate FRET) and ~90% (high FRET). Transitions between all FRET states were observed indicating that all conformations can interconvert (Supplementary Fig. 7). This is consistent with the burst analysis experiments (Fig. 6d). For the G193C–G309C mutant, the kinetic rates were similar in the presence of Hsp70 and/or Hsp90. When the interaction of Hsp70 with either TPR1 or TPR2B is weakened by a mutation, the kinetic rates for several of the transitions increased when both Hsp70 and Hsp90 were present. The faster dynamics was also observed for Hsp70 alone in the G193C–G309C N435A mutant, whereas, as expected, the kinetics of all three constructs were similar in the presence of Hsp90 alone. Taken together, dynamics between at least three conformations of Sti1 were observed in the presence of Hsp70 and/or Hsp90, and all conformations can interconvert. In addition interactions of Hsp70 with both TPR1 and TPR2B decrease the rate of the dynamic transitions.

Figure 6 | Deletion of the linker alters ternary complex formation. (a,b) Formation of ternary complexes between γHsp70, γHsp90 and either (a) wild-type Sti1 or (b) Sti1 Δlinker was visualized by analytical ultracentrifugation. The 0.5 μM fluorescein-labelled γHsp70 and 3 μM γHsp90 were incubated with 0.5 (black), 1 (navy), 2 (blue), 3 (cyan), 4 (wine), 6 (pink) and 8 μM Sti1 (light magenta) variant in 10 mM potassium phosphate at pH 7.5. Centrifugation was performed at 20 °C and 42,000 r.p.m. Sedimentation profiles were converted into dc/dt plots according to the standard procedures and fitted with bi-Gaussian functions. For clarity, only fits are shown. The s.e. values were below 1%. (c) Formation of ternary complexes between γHsp70, γHsp90 and wild-type Sti1 (black), Sti1 Δlinker N39A (red), Sti1 N39A (blue) and Sti1 Δlinker (pink) visualized by analytical ultracentrifugation. The 0.5 μM fluorescein-labelled γHsp70 and 3 μM γHsp90 were incubated with 3 μM Sti1 variant in 10 mM potassium phosphate at pH 7.5. Centrifugation was performed at 20 °C and 42,000 r.p.m. Sedimentation profiles were converted into dc/dt plots according to the standard procedures and fitted with bi-Gaussian functions. For clarity, only fits are shown. The s.e. values were below 1%.
Figure 6 | SpFRET measurements of fluorescently labelled Sti1. (a) The secondary structure of the different domains of Sti1. The residues mutated to a cysteine are shown in red (grey: not resolved in the structure). (b) Scheme of the different cysteine double mutants. The star marks the position of the fluorescent dye. (c) SpFRET efficiency plots of the mutants in panel (b). The 20 pM Sti1 was measured alone or mixed together with 10 μM Hsp90 or/and 25 μM Hsp70. The area under the curves was normalized. (d) FRET efficiency for the G193C-G309C mutant versus fluorescence lifetime of the donor in the presence of the acceptor. Multiple populations can be observed. The solid red line describes the expected relationship between donor lifetime and FRET efficiency when the populations are static, whereas the dashed green line indicates the theoretical curve when molecules undergo dynamic transitions between populations within a burst. In the absence of Hsps, the bursts fall symmetrically about the static line. In the presence of Hsps, a small deviation from the static line is observed for part of the population, suggesting there may be dynamic transitions between populations during a burst. (e) Representative data from spFRET TIRF experiments. Time traces of the donor and acceptor intensity (upper graph), total intensity corrected for the differences in sensitivity between the donor and acceptor channels ($I_T = \gamma I_D + I_A$ where $\gamma$ is the detection correction factor, middle graph) and FRET efficiency (lower graph) of the G193C–G309C Sti1 mutant in the absence and presence of Hsp70 and Hsp90. Left: a representative spFRET trace for the G193C–G309C Sti1 mutant in the absence of Hsps. Most of the traces of Sti1 in the absence of Hsps show a static FRET efficiency until either the donor or acceptor molecule photobleaches. In this case, the acceptor fluorophore photobleached first. Right: representative spFRET traces for the G193C–G309C Sti1 mutant in the presence of 25 μM Hsp70 and 10 μM Hsp90. A dynamic FRET signal is detectable in a significant fraction of the measured molecules. The fluctuations in the donor and acceptor fluorescence intensities indicate changes in the FRET efficiency due to the movement of Sti1 between different conformations. The donor fluorophore photobleached before the acceptor molecule and the total intensity dropped to background levels (see also Supplementary Fig. 6).
Discussion

Sti1/Hop connects Hsp90 and Hsp70, the two major chaperone machineries in the eukaryotic cell\textsuperscript{19,20}. Our analyses reveal that Sti1 consists of two structured modules with defined functions connected by a flexible linker. Of special interest is the C-terminal domain of Sti1 that consists of two structured modules with defined functions, which exhibit limited inter-domain flexibility. This is particularly important, as the DP2 module plays a regulatory role in the TPR domain. Thus, all three domains are strongly coupled and exhibit limited inter-domain flexibility. This is particularly important, as the DP2 domain has a significant impact on client activation \textit{in vivo}\textsuperscript{22,30–32}. In contrast, the N-terminal module consisting of the TPR1 domain followed by DP1 is not a rigid unit. Rather, these domains are flexible concerning their orientation. It is tempting to speculate that the interaction of TPR1–DP1 with client-bound Hsp70 may differ compared with TPR2B–DP2. The third element in Sti1 is the long linker between the two TPR modules. As expected, it is flexible and unstructured. It provides dynamics in the relative orientation of the TPR modules, which has significant functional consequences such as effects on the formation of complexes with Hsp70 and Hsp90. The fact that the flexible linker in Sti1 plays a regulatory role is reminiscent of what is known about the linker in Hsp90 that connects the N-domain with the M-domain. In Hsp90, this linker also provides regulatory sites that are proposed to modulate Hsp90 in a client- and co-chaperone-dependent manner\textsuperscript{41}. The deletion of the linker in Sti1 reduces the activation of the client GR by 50%. These effects result from the higher affinity of the Sti1 linker construct for Hsp70, which may be due to the closer distance between the alternative Hsp70-binding sites in Sti1 or a change in the dynamics of the protein.

Taken together, our results suggest that the linker acts as a molecular rheostat that, together with Hsp90, fine-tunes the affinity of Hsp70 for Sti1. The Hsp70–Hsp90–Sti1 chaperone machine is highly dynamic and needs to assemble and disassemble in a timely and ordered manner. Thus, slight changes in the binding affinities have a significant impact on the \textit{in vivo} performance of this molecular machine.

Both TPR–DP modules contain an Hsp70-binding site, TPR1 and TPR2B. The presence of two TPR domains that alternatively bind Hsp70 was puzzling, especially, as some Sti1/Hop homologues lack the TPR1–DP1 module\textsuperscript{22,30–32}. Although TPR1 is implicated in the interaction with Hsp70 (\textit{refs} 29,44,45), it seems functionally obsolete as the activation of clients \textit{in vivo} is not affected by its deletion\textsuperscript{22}. TPR2B represents the high-affinity interaction site for Hsp70 in the absence of Hsp90. However, we see pronounced effects of the TPR1 domain \textit{in vitro} in the presence of Hsp90. However, when TPR1 was inactivated in this construct, client activation was increased.

| Table 2 | Percentage of dynamic traces in total internal reflection fluorescence microscopy measurements. |
|-----------------|-----------------|-----------------|
|                 | G193C–G309C     | G193C–G309C     | G193C–G309C     |
|                 | N435A dynamic    | N39A dynamic    | N435A dynamic    |
| No Hsp          | 17              | 17              | 13              |
| + Hsp70         | 28              | 15              | 27              |
| + Hsp70 + Hsp90 | 28              | 21              | 23              |

| Table 3 | FRET efficiency values extracted from the HMM analysis of the TIRF measurements. |
|-----------------|-----------------|-----------------|
|                 | Low             | Intermediate    | High            |
| G193C–G309C     |                 |                 |                 |
| + Hsp70         | 0.16            | 0.52            | 0.84            |
| + Hsp90         | 0.24            | 0.66            | 0.92            |
| + Hsp70 + Hsp90 | 0.23            | 0.57            | 0.86            |
| G193C–G309C N39A|                 |                 |                 |
| + Hsp70         | 0.18            | 0.62            | 0.92            |
| + Hsp90         | 0.10            | 0.58            | 0.91            |
| + Hsp70 + Hsp90 | 0.14            | 0.58            | 0.91            |
| G193C–G309C N435A|                |                 |                 |
| + Hsp70         | 0.18            | 0.65            | 0.93            |
| + Hsp90         | 0.20            | 0.57            | 0.92            |
| + Hsp70 + Hsp90 | 0.17            | 0.57            | 0.92            |

FRET = Förster resonance energy transfer; HMM = Hidden Markov model; TIRF = total internal reflection fluorescence microscopy.

| Table 4 | Transition rates (in s\textsuperscript{-1}) extracted from the HMM analysis of the TIRF measurements. |
|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
|                 | Low \(\rightarrow\) | High \(\rightarrow\) | Low \(\rightarrow\) | Intermediate \(\rightarrow\) | Intermediate \(\rightarrow\) | High \(\rightarrow\) |
| G193C–G309C     |                 |                 |                 |                 |                 |                 |
| + Hsp70         | 4.5 (83)        | 1.0 (49)        | 1.5 (64)        | 1.5 (69)        | 0.9 (48)        | 1.5 (53)        |
| + Hsp90         | 3.4 (36)        | 0.6 (35)        | 1.7 (79)        | 0.9 (69)        | 0.9 (22)        | 0.6 (22)        |
| + Hsp70 + Hsp90 | 4.3 (51)        | 0.9 (41)        | 1.0 (22)        | 0.7 (29)        | 1.0 (34)        | 0.8 (35)        |
| G193C–G309C N39A|                 |                 |                 |                 |                 |                 |
| + Hsp70         | 3.5 (31)        | 1.0 (19)        | 1.5 (20)        | 0.8 (19)        | 1.7 (23)        | 1.0 (18)        |
| + Hsp90         | 3.0 (52)        | 1.3 (27)        | 2.1 (28)        | 2.4 (36)        | 1.3 (43)        | 1.3 (39)        |
| + Hsp70 + Hsp90 | 5.0 (15)        | 4.1 (7)         | 3.1 (22)        | 2.3 (27)        | 3.0 (8)         | 1.9 (7)         |
| G193C–G309C N435A|                |                 |                 |                 |                 |                 |
| + Hsp70         | 2.5 (20)        | 2.3 (8)         | 3.8 (18)        | 1.5 (14)        | 3.3 (6)         | 2.5 (3)         |
| + Hsp90         | 2.7 (10)        | 0.8 (9)         | 2.2 (23)        | 1.8 (25)        | 1.7 (26)        | 1.6 (13)        |
| + Hsp70 + Hsp90 | 4.1 (32)        | 4.0 (19)        | 3.2 (26)        | 2.4 (26)        | 2.4 (19)        | 1.2 (19)        |

Abbreviations: HMM = Hidden Markov model; TIRF = total internal reflection fluorescence microscopy. The number of measured transitions is given in the parenthesis next to the respective rates.
These findings point to a functional interplay between the different elements of Sti1. Despite its low affinity, our experiments define TPR1 as an important binding site for Hsp70 in Sti1. SpFRET experiments revealed that Hsp90 induces a more open conformation of Sti1 in which the two TPR–DP modules are further apart, increasing the accessibility of TPR1. Moreover, Hsp90 binds to TPR2A–TPR2B with a large interaction surface including an interaction of TPR2B with the Hsp90 M-domains\textsuperscript{22}. This reduces the accessible space for binding of Hsp70 to TPR2B and would favour the binding of Hsp70 to TPR1. This explains why in previous work a change in affinity for Hsp70 was observed in the presence of Hsp90 (refs 12,36,43).

The presence of two Hsp70-binding sites that are differentially activated and regulated suggests that Hsp70 switches between these two sites during client activation. Strikingly, we observed increased dynamics between the two Sti1 TPR-DP modules upon addition of Hsp90 and Hsp70. The induced dynamics often brings the two Hsp70-binding domains (TPR1 and TPR2B) into close proximity. This would facilitate the transfer of Hsp70 between TPR1 and TPR2B and explain the existence of the flexible linker/hinge region. The function of the linker seems tightly connected to the interaction with Hsp70 as the effects of the deletion of the linker (for example, the decreased formation of tightly connected to the interaction with Hsp70 as the effects of the deletion of the linker (for example, the decreased formation of tightly connected to the interaction with Hsp70). The induced dynamics between TPR1 and TPR2B would favour the binding of Hsp70 to TPR1. This explains why in previous work a change in affinity for Hsp70 was observed in the presence of Hsp90 (refs 12,36,43).

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potassium phosphate (pH 7.5). To determine the size of the complexes, the raw data were converted to d/c/d profiles as described before26 and then analysed by bi-Gaussian or tri-Gaussian functions.

The determination of the fractional coefficient for Sti1 and Alinker Sti1 was based on sedimentation experiments. The protein was detected using an absorbance optical system at 280 nm. Data analysis was performed using the c(S)-modul of UltraScan27.

**Surface plasmon resonance spectroscopy**. SPR measurements were carried out with a BiacoreXTM instrument (GE Bioscience). Hsp70 or Hsp90 were covalently linked to a CM5 SPR chip deploying amine-coupling reagents, as described by the manufacturer. The analyte was flushed over the chip in 40 mM Hepes (pH 7.5), 20 mM KC1, 5 mM MgCl2, 0.005% (v/v) Tween with a constant flow of 10 µl min-1 at a temperature of 20 °C. The SPR signal change on injection was subtracted by the reference signal and plotted against the corresponding concentration. The direct binding signal was analysed by fitting the binding curves to a Langmuir equation. Error bars indicate s.e.

**Solution-based single-pair FRET experiments**. For the single-pair FRET measurements, a home-built set-up with pulsed interleaved excitation and multi-parameter fluorescence detection28 was used29. By using pulsed interleaved excitation-multiparameter fluorescence detection, we maximize the information gathered from the sample and can separate subpopulations based on fluorescence lifetime, anisotropy or labelling stoichiometry31. The fluorescently labelled Sti1 was diluted to a concentration < 40 pM in 50 mM Hepes (pH 7.5), 150 mM KC1 and 10 mM MgCl2 and measured alone or in the presence of 25 µM Hsp70 and/or 10 µM Hsp90. At each dye concentration, the average molecule in the probe volume is much less than one. Analysis was performed with our in-house PAM software written in the MATLAB language (Mathworks). In the first step, all molecules with both dyes were selected and sorted in a FRET efficiency histogram. To look for the dynamic behavior, the data were fitted to a three state model. The structure of Sti1 TPR2A-TPR2B-DP2 was modelled using the program INSTAR54 and GIFT55. The forward scattering, Rg, the maximum dimension, Dmax, and the inter-atomic distance distribution functions, (P(r)), were calculated with the program GNOM. The masses of the solutes were evaluated by comparison of the forward scattering intensity with that of a human serum albumin reference solution (molecular mass 69 kDa). EOM calculations were carried out using the EOM program56 and using default settings. A random pool of 100,000 independent structures was generated using the primary sequence and the available structures of the individual domains as input (TPR1, DP1, TPR2-A, TPR2-B, DP2). The unordered regions in the termini and the structured domains were randomized. Using the same genetic algorithm and using the default settings, a subset of a few independent structures was selected that describes the experimental SAXS best and used to prepare the figures showing Rg/Dmax distributions and structural ensembles.

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Author contributions
A.R., D.W., S.L., F.T., M.H., J.H., G.H., K.R. and A.B.S. performed experiments. A.R., H.K., J.B. and D.C.L. designed experiments. T.M. performed and analysed the SAXS experiments. A.R., J.B. and D.C.L. wrote the manuscript. J.B. conceived the project.

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