Supplementary methods:

Untagged CHIP purification
pET3a-CHIP was transformed into BL21-DE3 star cells and grown in LB supplemented with 100 μg/ml ampicillin. At an OD₆₀₀nm of 0.4, CHIP expression was induced by the addition of IPTG to a final concentration of 1 mM for 3-4 h at 30°C. The culture was then spun at 6000 g for 20 min at 4°C, pellet resuspended in 50 mM Tris (pH 8) and 10% sucrose (10 ml per pellet from 1 l culture), snap-frozen in liquid nitrogen as 1 ml aliquots and stored overnight at -80°C. The following day, the aliquots were thawed and supplemented with KCl, lysozyme, benzamidine, DTT and pefabloc to final concentrations of 150 mM, 2 mg/ml, 1 mM, 1 mM and 1 mM respectively, after which they were incubated on ice for 30 min. The aliquots were frozen in liquid nitrogen, promptly thawed and sonicated 2X for 10 sec with 10 sec incubations on ice in between. Samples were then spun at 16000 g for 20 min at 4°C and supernatant (lysate) collected. The lysate was loaded onto a DEAE-sepharose column equilibrated with Buffer A (50 mM HEPES, pH 7.5, 50 mM NaCl, 10% glycerol, 0.01% Triton X-100, 0.1 mM EDTA, 1 mM DTT and 1 mM benzamidine), washed with 5 CV Buffer A and then eluted over 6 CV using a linear salt gradient (up to 1 M NaCl). CHIP-containing fractions were pooled, dialysed into Buffer A and concentrated using spin concentrators (Millipore) if required or loaded directly onto the next column using a super-loop (GE healthcare). The pooled DEAE fractions were then loaded onto an SP-HP column (GE healthcare) equilibrated in Buffer A, washed with 5 CV Buffer A and eluted over 15 CV using a linear salt gradient as above. Purified CHIP-containing fractions were snap-frozen in liquid nitrogen and stored at -80°C.

MD simulations
Crystallographic water molecules were retained in all simulations, which were carried out using the AMBER (Assisted Model Building Refinement) 9 package (http://ambermd.org/) together with the ff99SB forcefield. The antechamber and LEaP modules were used to set up the simulation. The N termini of the CHIP protein (Chain A) and the N and C-termini of the peptide (Chains E, F) were capped using ACE and NME caps respectively. Systems were solvated in a TIP3P water box with walls at least 8 Å away from any protein atom and net charges on the protein were neutralized using counter ions as required (20-26 Na⁺). A brief energy minimization was carried out followed by heating of the systems to 300K. Subsequent MD simulations were performed under constant pressure and temperature using the Sander module. All simulations were run for 20 ns and analyzed using the ptraj module of AMBER 9 (http://ambermd.org/) and the Bio3D R package. Figures were prepared using PyMOL v1.4.1 (http://www.pymol.org).

For simulations of CHIP wt with Hsp70 peptide and CHIP wt with Hsp90 peptide bound to only one CHIP monomer, the sequence of the Hsp70 peptide ‘SGPTIEEVD’ was modelled onto the Hsp90 peptide (Chains E, F) in the 2C2L crystal structure using PyMOL v1.4.1. A 20 ns MD simulation was run on the CHIP dimer (Chains A, B) with Hsp70 peptides bound to both CHIP monomers. Additionally a 20 ns simulation of the CHIP dimer with Hsp90 peptide bound to one monomer (Chains A, B, F from 2C2L) was carried out. The preparation of files and MD simulations were carried out as described above and in the main methods.
**SEC and Hydrodynamic analysis of purified full-length CHIP**

Size exclusion chromatography (SEC) was performed on a Superdex 200 10/300 GL column. The column was equilibrated with 1.5 CV of buffer (50 mM HEPES, pH 8, 100 mM NaCl, 1 mM DTT) at a flow rate of 0.5 ml/min, following which the sample was loaded and eluted with a further 1.5 CV. SEC experiments were also performed on a Superdex 75 16/60 column with a 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1 mM EDTA and 5 mM DTT buffer system. Hydrodynamic properties were calculated by dynamic light scattering (DLS) at 10°C on a Zetasizer APS (Malvern Instruments, UK) equipped with a 50-mW laser light source (830 nm). Scattering data were collected at a scattering angle of 90° for 10 s, repeated at least 12 times, and averaged. The experiments were repeated in triplicate. Autocorrelation data were fit to a model of a multiple-exponential form suitable for polydisperse solutions using the protein-specific software supplied with the instrument.
**Supplemental Figure, Table and movie legends:**

**Fig S1:**

**A.** Peptide binding assay showing His-Ctip wt binding to the indicated biotin-labelled peptides based on the C-terminus of Hsp70 (wt: GPTIEEVD, mut: GAAEEVD) that were immobilised on a streptavidin-coated microtitre plate. Binding was quantified using anti-His mAb and enhanced chemiluminescence.

**B.** Binding assay showing His-Ctip wt or ΔTPR binding to the indicated peptides as in A.

**C.** *Upper panel*: Binding assay showing His-Ctip wt binding to a series of Hsp70-based peptides where the individual amino acids were sequentially substituted by Ala residues and analyzed as in A. *Lower panel*: Immunoblots showing *in vitro* ubiquitination assays with His-Ctip as the E3 ligase and IRF-1 as the substrate. The assays were carried out in the presence (and absence) of either peptide carrier (DMSO) or Ala-scan Hsp70 peptides as in the upper panel (10 μM).

**D.** Binding assay showing His-Ctip wt binding to Hsp70 wt peptide (SGPTIEEVD) or Hsp70 peptide where the C-terminal Asp residue has been substituted with Ala, compared to a peptide based on the C-terminus of Hsp90 (DTSRMEEVD).

**Fig S2:**

**A.** Untagged CHIP wt (or CHIP K30A) expression was induced in *BL21-DE3* star cells using 1 mM IPTG (see supplementary text S1 for details). The majority of the over-expressed protein was found to be soluble (left panel). *BL21-DE3* star lysate containing CHIP was then loaded onto a DEAE-Sepharose column and eluted over a salt gradient (up to 1 M NaCl). CHIP-containing fractions (middle panel) were pooled, loaded onto an SP-HP column and eluted over a salt gradient as above (right panel). Eluates from the SP-HP column were used in the assays described in this manuscript. *Red arrows* indicate the untagged CHIP protein. CHIP wt and K30A behaved in a similar manner during purification.

**B.** Normalization of His-Ctip wt and K30A protein levels. A titration of His-Ctip wt or K30A was coated on a microtitre plate to normalize protein levels on the well. Protein levels were quantified by luminometry using an anti-His mAb and HRP-tagged anti-mouse antibody. Untagged CHIP wt and K30A were normalized in a similar manner and quantified using anti-CHIP antibody.

**C.** Dynamic light scatter data for wt and K30A CHIP. The Stokes radius of wt CHIP was 4.67 nm and K30A mutant 4.89 nm (distribution by volume). These sizes are consistent with a predominantly dimeric sample. The crystal structure of dimeric murine CHIP (amino acids 24-304; 2C2L.pdb) has a calculated Stokes’s radius of 4.12 nm; max dimension 11.4 nm.

**D.** Size exclusion chromatography for wt and K30A CHIP where the absorbance in arbitrary units is plotted against time in min.

**Fig S3:**

**A.** InstantBlue stained gel of His-Ctip wt (*left panel*) or K30A mutant (*right panel*) following limited proteolysis with Glu-C protease. FL is the full-length protein and band 1 is a cleavage product that persists in the K30A mutant. Band 2 is only observed in digests of the wt protein. **B.** Colloidal Blue stained gel of His-Ctip-K30A or wt protein following limited proteolysis with trypsin protease (*left panel*) or of His-Ctip wt protein in complex with wt or mutant Hsp70 peptides as indicated (*right panel*). FL, band 1 and band 2 are described in A. The digest for His-Ctip wt in complex with Hsp70 wt peptide closely resembles the digest of the K30A mutant.
Fig S4:
A. Root mean square fluctuation (RMSF) of Cα obtained from the trajectories of the 20 ns simulations of CHIP wt with one monomer (Chain A in the crystal structure 2C2L) unbound and one monomer (Chain B) bound to Hsp90 peptide. The score of the positional fluctuation analysis averaged over amino acid were colour coded and indicated on the crystal structure. B. Positional fluctuation (RMSF) scores of Cα averaged over the whole CHIP dimer in MD simulations are shown. CHIP wt shows a higher overall fluctuation than either the K30A mutant or any of the peptide bound forms. CHIP wt with Hsp90 peptide bound to only one monomer shows a decrease in fluctuation compared to apo CHIP wt, but an increase over CHIP-K30A or CHIP wt with Hsp90 peptide bound to both monomers. C. Root mean square fluctuation (RMSF) of Cα obtained from the trajectories of the 20 ns simulations of CHIP wt + Hsp70 peptide. The score of the positional fluctuation analysis averaged over amino acid was colour coded and indicated on the crystal structure (left panel). Snapshots after 20 ns simulation of CHIP wt dimer +/- Hsp70 peptide (right panel). Both fluctuation and overall movement of CHIP wt bound to the Hsp70 peptide (yellow) are significantly different from the simulation of apo CHIP (blue), showing less flexibility and a more compact structure that resembles the results of the Hsp90 peptide-bound CHIP dimer.

Fig S5:
Sequence coverage for wt and K30A CHIP was generated by a screenshot from Proteome Discoverer 1.4 software analysis.

Table 1:
Excel spreadsheet providing a list of peptides identified during MS analysis of wt and K30A CHIP generated using Proteome Discoverer 1.4 software.

Movie 1:
Overlay of 20 ns MD simulations of CHIP wt and CHIP K30A to illustrate the results in Figures 4 and 5.
Supplementary Figures

Figure S1

A

B

C

D

Peptide → CHIP

CHP wt

Hsp70 wt peptide binding (RLU)

GPTIEEVD

GAAAEEVD

Hsp70 wt peptide binding (RLU)

Hsp70 mut peptide binding (RLU)

D641A

V640A

E639A

E638A

I637A

T636A

P635A

G634A

S633A

Hsp70 Peptide binding (RLU)

Peptide → CHIP

Peptide → CHIP

HSP70 wt

CHIP binding (RLU)

Hsp70 wt

DMSO

Hsp70 D641A

Hsp90 a

C-terminal Hsp70 peptide derivatives

Ub-IRF-1

IRF-1
Figure S2

A

Anion Exchange: DEAE-Sepharose

Cation Exchange: SP-HP

B

CHIP detection (RLU)

CHIP (ng)

WT

Size Distribution by Volume

K30A

Size Distribution by Volume

D

Absorbance (mAU)

Time (min)
Figure S3

A

Figure S4

A

Dimeric CHIP wt with Hsp90 peptide bound to one monomer (left)

B

Table:

<table>
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<tr>
<th>Sample</th>
<th>Average fluctuation score</th>
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<td>CHIP wt</td>
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<tr>
<td>CHIP K30A</td>
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<tr>
<td>CHIP wt + Hsp90 peptide bound to both monomers</td>
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<tr>
<td>CHIP wt + Hsp90 peptide bound to one monomer</td>
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</tr>
</tbody>
</table>
Figure S5

A