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Research article

Swapping the linkers of canonical Hsp70 and Hsp110 chaperones compromises both self-association and client selection

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ABSTRACT

Plasmodium falciparum heat shock protein 70-1 (PfHsp70-1) and PfHsp70-z are essential cytosol localised chaperones of the malaria parasite. The two chaperones functionally interact to drive folding of several parasite proteins. While PfHsp70-1 is regarded as a canonical Hsp70 chaperone, PfHsp70-z belongs to the Hsp110 subcluster. One of the distinctive features of PfHsp70-z is its unique linker segment which delineates it from canonical Hsp70. In the current study, we elucidated the role of the linker in regulating Hsp70 self-association and client selection. Using recombinant forms of PfHsp70-1, PfHsp70-z and *E. coli* Hsp70 (DnaK) and their respective linker switch mutants we investigated self-association of the chaperones using surface plasmon resonance (SPR) analysis. The effect of the changes on client selectivity was investigated on DnaK and its mutant through co-affinity chromatography coupled to LC-MS analysis. Our findings demonstrated that the linker is important for both Hsp70 self-association and client binding.

1. Introduction

Hsp70 chaperones are crucial in maintaining cellular proteostasis. Hsp70s facilitate *de-novo* protein folding of nascent polypeptides, protein translocation, signal transduction and further prevent protein misfolding and aggregate formation [1–4]. Hsp70 domain architecture is typically characterised by an N-terminal nucleotide binding domain (NBD) and a C-terminal substrate binding domain (SBD) adjoined by a linker segment [5]. The Hsp70 SBD is further delineated into SBDα also termed the lid which closes over the SBDβ when bound to substrate. Generally, Hsp70s can be divided into canonical and non-canonical isoforms [5–7]. Canonical Hsp70s typically have the same structural organisation as *E. coli* Hsp70 (also known as DnaK). On the other hand, non-canonical Hsp70 cluster is characterised by members of the Hsp110 and Grp170 chaperones [6,8]. Although both canonical and non-canonical Hsp70s exhibit similar domain architecture, non-canonical Hsp70 members are distinguished from their canonical counterparts by the presence of extended acidic insertions in their substrate binding domains (SBDs) and linker segments [5,9]. The functional cycle of Hsp70 is regulated by nucleotides: ADP-Hsp70 exhibits higher affinity for substrate while ATP-Hsp70 possesses less affinity for substrate leading to its release. The linker of Hsp70 is known to transmit signals across the two domains of Hsp70, thus accounting for the allosteric function of the chaperone. We recently established that the distinct linker of the *Plasmodium falciparum* Hsp110 (PfHsp70-z,

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PF3D7_0708800) regulates the functional specialisation of this chaperone [10].

The cellular function of Hsp70 is regulated in part by the interconversion between the monomeric and oligomeric forms of the chaperone [11]. Thus, oligomerisation of Hsp70 is thought to regulate levels of the functionally active monomeric form of the chaperone. In addition, oligomerisation of Hsp70 is implicated in its interaction with functional partners such as Hsp40/J-proteins (co-chaperones) and Hsp90, a chaperone partner [12,13]. In this regard, the dimerisation of Hsp70 is necessary for its effective association with Hsp40 co-chaperone [11,13]. A recently described Hsp90-Hsp70-Hop complex revealed the presence of two Hsp70 molecules forming a dimer [12,14] and this may imply that Hsp70 dimerisation is required for the binding and folding of client

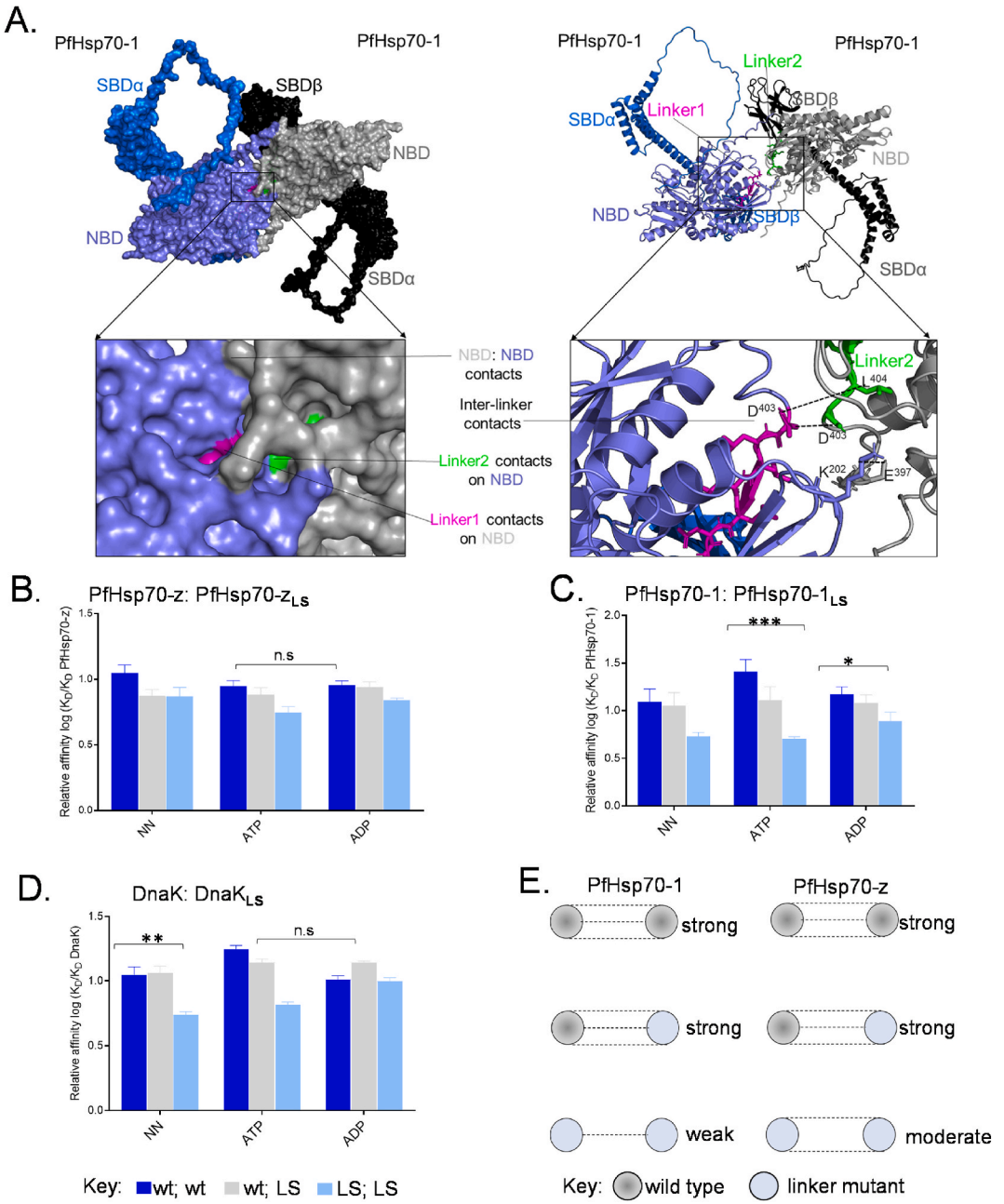

Fig. 1. Effects of linker changes on the self-association of Hsp70. A representative three-dimensional model generated by HADDOCK shows that the PfHsp70-1 linker makes contacts with the linker and the NBD of another PfHsp70-1 monomer during self-association (A). Self-association of wild type and mutant forms of PfHsp70-z (B), PfHsp70-1 (C) and DnaK (D) explored using SPR analysis was carried out in the absence of nucleotide (NN) or presence of ADP/ATP. The data represents 3 independent repeats of the assay. The statistical significance was determined by one way ANOVA at $p < 0.01$***. (E) A model demonstrating the respective roles of the linker of canonical Hsp70. The terms, ''wt'' and ''LS'' represent wild type proteins or their linker switch mutants, respectively.

proteins. Although oligomerisation in Hsp70s has been reported in several studies [15–17], the mechanism by which the process takes place remains elusive. Lebepe and colleagues (2020) [17], demonstrated that there is increased propensity for dimerisation of canonical Hsp70 when it is bound to ATP. While the linker of canonical Hsp70s has been implicated in oligomerisation of the protein [18], the contribution of this motif in the oligomerisation of canonical Hsp70 remains elusive. In addition, the function of the linker of Hsp110 in regulating oligomerisation remains unexplored. In the current study, we swapped the linker segments of canonical Hsp70s (*E. coli* DnaK [19] and *Plasmodium falciparum* Hsp70-1 [20]) and that of PfHsp70-z [3,10].

The collaborative interaction between canonical Hsp70s and their co-chaperones such as Hop and Hsp110 is crucial for their chaperone role [21,22]. We previously established that linker swap mutations between canonical and non-canonical Hsp70s compromises the association of Hsp70 chaperones with nucleotides and the co-chaperone, Hsp40 [10]. Hsp70's association with Hop occurs via the formers' strongly electronegative C-terminal EEVD motif that binds to the electropositive TPR domains of Hop [22–24]. PfHsp70-1 and PfHop have been shown to interact in a nucleotide regulated fashion (ADP enhances their association) [22]. On the other hand, ATP enhances the association of Hsp70 with its nucleotide exchange factor, Hsp110, through NBD:NBD contacts [25]. Since the linker is thought to dictate the overall conformation of Hsp70 [10], we enquired the role of this motif in regulating the self-association of PfHsp70-1, PfHsp70-z and DnaK. We further explored the role of the linker in modulating the hetero-association of PfHsp70-1 and PfHsp70-z. Finally, we explored the role of the linker swap on the interaction of DnaK with its cellular clients.

2. Results

2.1. The linker of canonical Hsp70 is more important for oligomerisation than that of Hsp110

First, we sought to investigate the effects of the linker swap mutations on the oligomerisation of Hsp70. Details on the construction of the Hsp70 linker switch mutants and their nomenclature are provided in the first segment of the Methods section. Bioinformatics predictions made using the Haddock server [26,27] suggest that the self-association of canonical Hsp70 is driven by docking of the linker of one of the monomers to the NBD or the SBDβ segment of the second monomer (Fig. 1a; Table S1). Notably, the linker substitutions led to switches of residues involved in the oligomerisation of the respective monomers in either wild type:mutant or mutant:mutant pairs (Figures S1-3; Table S1). To validate this, surface plasmon resonance (SPR) analysis was conducted. Notably, PfHsp70-z exhibited a high propensity to self-associate (K_D = 1.39 nM in *apo* state) and its oligomerisation was not regulated by nucleotide

Table 1
Kinetics for self-association of the Hsp70s and their linker switch variants.

Ligand	Analyte	K_D (M)
PfHsp70-z	PfHsp70-z (NN)	1.39 (±0.09)e^{-9}
	PfHsp70-z (ADP)	2.93 (±0.03)e^{-9}
	PfHsp70-z (ATP)	9.08 (±0.08)e^{-9}
PfHsp70-z$_{LS}$	PfHsp70-z (NN)	3.17 (±0.07)e^{-9}
	PfHsp70-z (ADP)	2.79 (±0.09)e^{-9}
	PfHsp70-z (ATP)	1.84 (±0.04)e^{-9}
PfHsp70-z$_{LS}$	PfHsp70-z$_{LS}$ (NN)	2.75 (±0.05)e^{-9}
	PfHsp70-z$_{LS}$ (ADP)	9.66 (±0.06)e^{-9}
	PfHsp70-z$_{LS}$ (ATP)	1.89 (±0.09)e^{-8}
PfHsp70-1	PfHsp70-1 (NN)	3.75 (±0.05)e^{-6}***
	PfHsp70-1 (ADP)	1.29 (±0.09)e^{-6}
	PfHsp70-1 (ATP)	2.68 (±0.08)e^{-7}
PfHsp70-1$_{LS}$	PfHsp70-1 (NN)	4.70 (±0.70)e^{-6}
	PfHsp70-1 (ADP)	3.37 (±0.07)e^{-6}
	PfHsp70-1 (ATP)	9.98 (±0.08)e^{-7}
PfHsp70-1$_{LS}$	PfHsp70-1$_{LS}$ (NN)	4.24 (±0.04)e^{-5}***
	PfHsp70-1$_{LS}$ (ADP)	1.72 (±0.02)e^{-5}
	PfHsp70-1$_{LS}$ (ATP)	5.90 (±0.90)e^{-5}
DnaK	DnaK (NN)	2.28 (±0.08)e^{-6}***
	DnaK (ADP)	1.89 (±0.09)e^{-6}
	DnaK (ATP)	5.04 (±0.04)e^{-7}
DnaK$_{LS}$	DnaK (NN)	2.02 (±0.02)e^{-6}
	DnaK (ADP)	1.13 (±0.03)e^{-6}
	DnaK (ATP)	9.03 (±0.03)e^{-8}
DnaK$_{LS}$	DnaK$_{LS}$ (NN)	2.97 (±0.07)e^{-5}***
	DnaK$_{LS}$ (ADP)	2.05 (±0.05)e^{-6}
	DnaK$_{LS}$ (ATP)	5.40 (±0.40)e^{-6}

*The assays were conducted in the absence of nucleotides (NN) or in the presence of 5 mM ATP/ADP. The data represents assays conducted in duplicates and from 3 independent runs. Standard errors are shown in brackets. The statistical significance was determined by one way ANOVA. *** The asterisks denote significance at $p < 0.01$ relative to the self-association of the respective wild type protein as determined in the absence of nucleotides.

(Fig. 1, Table 1). In addition, its linker mutant, PfHsp70-z_{LS}, demonstrated capability to self-associate, albeit, with reduced affinity (K_D = 2.75 nM in *apo* state; Table 1; $p > 0.01$) relative to the wild type protein. Interestingly, in the ATP-state, PfHsp70-z_{LS} demonstrated higher propensity to self-associate (approximately 10-fold) than in *apo*- or ADP-states (Table 1; Table S2). Furthermore, PfHsp70-z_{LS} retained the same level of affinity for the wild type protein (PfHsp70-z) (Fig. 1; Table 1).

It has been proposed that ATP promotes oligomerisation of canonical Hsp70 [11,13,17,28]. In agreement with this, the oligomerisation of PfHsp70-1 occurred at comparable affinity in the absence of nucleotide and in the presence of ADP (Table 1; Fig. 1). Additionally, ATP induced a tenfold increase in PfHsp70-1 self-association compared to either the *apo*- or ADP-states. (Table 1; Fig. 1, panel C). Interestingly, the association of PfHsp70-1 and its mutant, PfHsp70-1_{LS}, followed a similar trend (Table 1; Fig. 1, panel C). Notably, the self-association of PfHsp70-1_{LS} was significantly lower (K_D value within millimolar range) compared to that of either

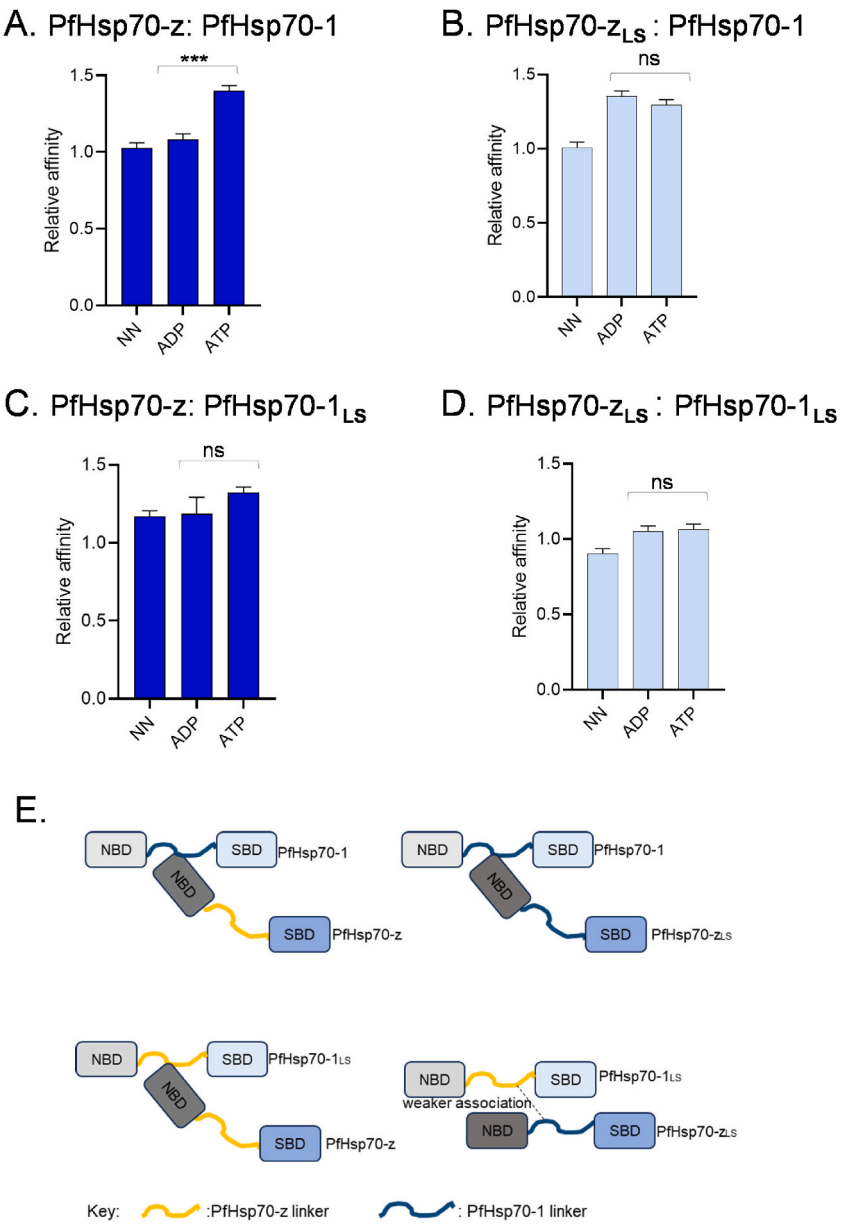

Fig. 2. SPR analysis demonstrated the role of the linker in the heterologous association of PfHsp70-1 and PfHsp70-z. The bar graphs were generated using relative K_D values obtained by SPR analysis. Assays were conducted in the *apo*- (NN) or ADP/ATP-states (A–D). Estimated affinities were calculated relative to the affinity of PfHsp70-1: PfHsp70-z (NN). The data represents 3 independent repeats of the assay. The statistical significance was determined by a one way ANOVA test at $p < 0.01$***. (E) Proposed model highlighting the respective roles of linkers in PfHsp70-z: PfHsp70-1 interaction.

PfHsp70-1:PfHsp70-1 or PfHsp70-1:PfHsp70-1_{LS}. This suggests that substitution of the linker of PfHsp70-1 by that of PfHsp70-z abrogated self-association of the chaperone as the derived K_D values in the millimolar range denote a requirement for high molecular concentrations to establish functional association (Table 1). In addition, the self-association of PfHsp70-1_{LS} was nucleotide independent. This confirms the rigidity of the PfHsp70-z linker as previously reported [10].

To further confirm the role of the linker of canonical Hsp70 in self-association, we substituted the linker of the *E. coli* DnaK with that of PfHsp70-z. We observed that DnaK:DnaK and $DnaK_{LS}$:DnaK interactions generally followed the same pattern (Table 1; Fig. 1, panel

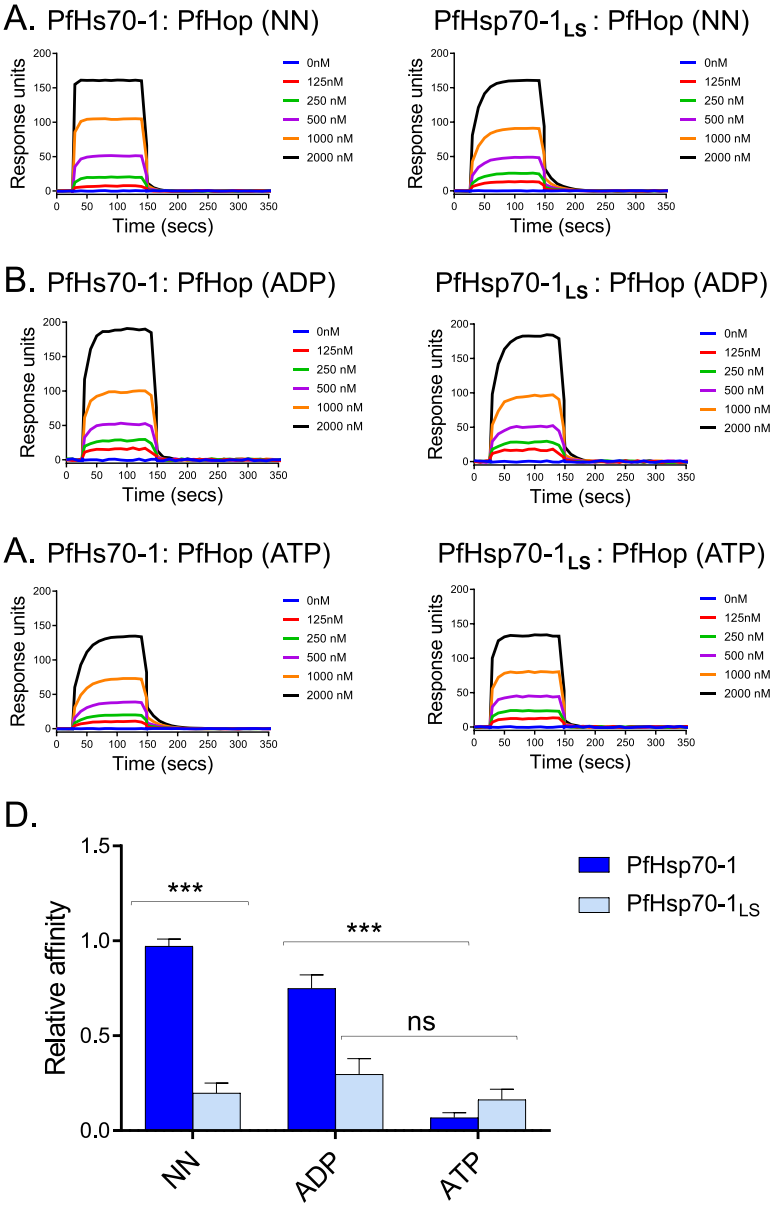

Fig. 3. The linker of PfHsp70–1 is important for interaction with the co-chaperone, PfHop. The SPR sensorgrams show the concentration-dependent association between immobilized PfHsp70-1/PfHsp70-1_{LS}, and the analyte, PfHop. The assays were conducted under the following conditions: apo state (A); 5 mM ADP (B); and 5 mM ATP (C). Additionally, the relative affinities for PfHsp70-1_{LS}'s association with PfHop were calculated relative to the affinity of wild type PfHsp70-1 for PfHop, as determined in the *apo*-state (D). The data represents three independent assay runs. Statistical significance was determined using a one-way ANOVA test with $p < 0.01$***.

D) as we observed for PfHsp70-1 and its mutant, PfHsp70-1_{LS}. In addition, in the presence of ATP, $DnaK_{LS}$ registered higher affinity for self-association than wild type protein (10-fold higher). This demonstrates that the contribution of the linker of PfHsp70-z was not the same in PfHsp70-1_{LS} as compared to $DnaK_{LS}$. This suggests that besides the linker, other segments of Hsp70 are important for its oligomerisation. Overall, we propose that the linker regulates the self-association of canonical Hsp70 and that ATP augments this process (Fig. 1E). That PfHsp70-z_{LS} demonstrated reduced propensity for self-association in the presence of ATP as opposed to the wild type protein whose self-association was nucleotide independent further highlights the functional distinctions of the linker of canonical Hsp70 versus that of Hsp110 (Fig. 1, Table 1).

2.2. The linkers of PfHsp70-z and PfHsp70-1 are important for the heterologous association of the two chaperones

Previously, we showed that the linker segments of canonical Hsp70s and that of PfHsp70-z are crucial for binding to nucleotides, peptide substrates, and J protein/Hsp40 [10]. Additionally, we previously demonstrated that PfHsp70-1 and PfHsp70-z directly interact, and ATP substantially enhances their association [3] (Table S2). PfHsp70-z is thought to serve as a nucleotide exchange factor of PfHsp70-1 and hence their association is important for the chaperone cycle of PfHsp70-1. Against this backdrop, we explored the influence of the linker swaps on the heterologous association of the two chaperones.

Generally, in the apo- and ADP-states the interaction of PfHsp70-1 and PfHsp70-z occurred within the same order of affinity (Table S2; Fig. 2, panel A; Figure S4). However, as previously reported [3], ATP enhanced the direct association of the two chaperones. Notably, we observed that both ADP and ATP enhanced the PfHsp70-z_{LS}:PfHsp70-1 association relative to the scenario in which nucleotides were absent (Fig. 2, panel B). This shows that the presence of the linker of canonical Hsp70 in PfHsp70-z_{LS} subjected this protein to conformational modulation by both ADP and ATP, possibly leading the protein to assume a conformation that favours association with PfHsp70-1. Interestingly the association of PfHsp70-z with PfHsp70-1_{LS} was not sensitive to nucleotides (Fig. 2, panel C). This implies that the incorporation of the rigid linker of PfHsp70-z into PfHsp70-1_{LS} rendered the latter relatively insensitive to nucleotide, as previously evidenced by intrinsic fluorescence analysis of the protein that was conducted in the presence of either ADP or ATP [10]. Notably, the linker switch mutants (PfHsp70-1_{LS}: PfHsp70-z_{LS}) showed relatively lower affinity for one another when compared to their wild type proteins (PfHsp70-1:PfHsp70-z) (Table 1; Fig. 2, panel D; Figure S4). This demonstrates that the original linkers of both proteins are required to facilitate the association of the two proteins. However, in the presence of either ATP or ADP, the K_D values that represent the association of the two mutant proteins dropped by one order of magnitude (Table S3) suggesting that this interaction was modulated by nucleotides in atypical fashion since both ATP and ADP modulated the association of the two proteins in similar fashion. In summary, our findings indicate that the linker of PfHsp70-z plays a less important role in its interaction with its functional partner, PfHsp70-1. Conversely, the linker of PfHsp70-1 is important for its interaction with PfHsp70-z. Overall, PfHsp70-1's association with PfHsp70-z in the presence of ATP (known to promote their association) requires that at least one of the two proteins possess its original linker (Fig. 2E).

2.3. PfHsp70-1_{LS} exhibits reduced affinity for the co-chaperone, PfHop

PfHop is a co-chaperone of PfHsp70-1 that binds to the C-terminal EEVD motif of the chaperone [22,29]. PfHop acts as a module facilitating the assembly of a functional complex comprising PfHsp70-1 and its chaperone partner, PfHsp90 [22,29]. The current study examined the impact of the linker swap on PfHsp70-1_{LS}'s ability to directly bind to PfHop.

As expected, PfHsp70-1 interacted with PfHop at nanomolar concentrations (Table S4) as previously established [22]. In both the *apo*- and ADP-states, PfHsp70-1 exhibited comparable affinities for PfHop as expected [22] (Fig. 3A–D, Table S3). Furthermore, upon the addition of ATP, PfHsp70-1's affinity for PfHop was diminished by a 24-fold magnitude with K_D value shifting from 3.89 nM to 94.6 nM, aligning with findings from an earlier study [22] (Table S3). Generally, in *apo*- and ADP-states, PfHsp70-1_{LS} exhibited lower affinity (by approximately 10-fold) for PfHop than the wild type PfHsp70-1 protein (Table S4). In addition, the K_D values recorded for PfHsp70-1_{LS}:PfHop interaction fell within the same order of magnitude (Table S3; Fig. 3D), irrespective of nucleotide status. This observation further reflects that the incorporation of the linker of PfHsp70-z into PfHsp70-1_{LS} imparted conformational rigidity to the mutant protein, rendering it insensitive to nucleotide, as previously suggested [10]. In addition, this reduced conformational flexibility may account for its reduced affinity for PfHop (Fig. 3A–D).

2.4. The canonical Hsp70 linker is more important for substrate binding than that of Hsp110

Approximately 24 % of the *P. falciparum* proteome is enriched in asparagine (N, Asp) and glutamine (Q, Glu) [30]. As such, the proteome of this malaria parasite is predicted to be aggregation prone. It has further been proposed that Hsp70 chaperones of the parasite are tailored to facilitate fold of the potentially unstable proteome of the parasite [17,24]. In line with this hypothesis, we previously explored the substrate binding preferences of PfHsp70-1 and PfHsp70-z relative to those of their linker switch mutants [10]. Using a peptide model, GFRLLLMYRF, and its Asp-enriched variant, GFRNNNMYRF, we established that both PfHsp70-1 and PfHsp70-z preferentially bind to the Asp-enriched peptide. In addition, we noted that while PfHsp70-1's affinity for GRFLLLMYRF and

GRFNNNMYRF was regulated by ADP/ATP, the binding of PfHsp70-z to the peptides was nucleotide independent. Furthermore, we observed that while the affinity of PfHsp70-1 for GRFLLLMYRF and GRFNNNMYRF was influenced by nucleotide, the propensity of PfHsp70-z to bind to the peptides was independent of nucleotide [10]. Furthermore, we observed that of the two linker switch mutants only PfHsp70-1_{LS} exhibited reduced affinity for the peptides relative to the wild type chaperone. In the current study, we expanded the study by exploring how PfHsp70-1 and PfHsp70-z interact with two additional peptides (NRLLTG and ALLLMYRR) which were described before [24]. We further explored the interaction of the chaperones and their linker switch mutants with the Asp-enriched forms (NRNNTG and ANNNMYRR) of the two peptides. Furthermore, we explored the interaction of the peptides with *E. coli* DnaK and its linker switch mutant (DnaK$_{LS}$), harbouring the PfHsp70-z linker.

PfHsp70-z generally exhibited higher affinity for the peptide, ANNNMYRR, than for ALLLMYRR confirming the previously reported preference of plasmodial Hsp70s for asparagine enriched peptides [10,17,24]. In addition, PfHsp70-z demonstrated higher affinity for ANNNMYRR than for the NRLLTG/NRNNTG pair (Fig. 4, panel A). Notably, the affinity of PfHsp70-z for the peptides NRLLTG/NRNNTG was generally within the same order of magnitude irrespective of the nucleotide state (Fig. 4A; Table S4), confirming that this protein has restricted allosteric function as previously proposed [10]. Generally, PfHsp70-z_{LS} demonstrated a higher affinity for the peptides than the wild type protein. Surprisingly, PfHsp70-z_{LS}'s affinity for the peptide, ANNNMYRR, was enhanced by

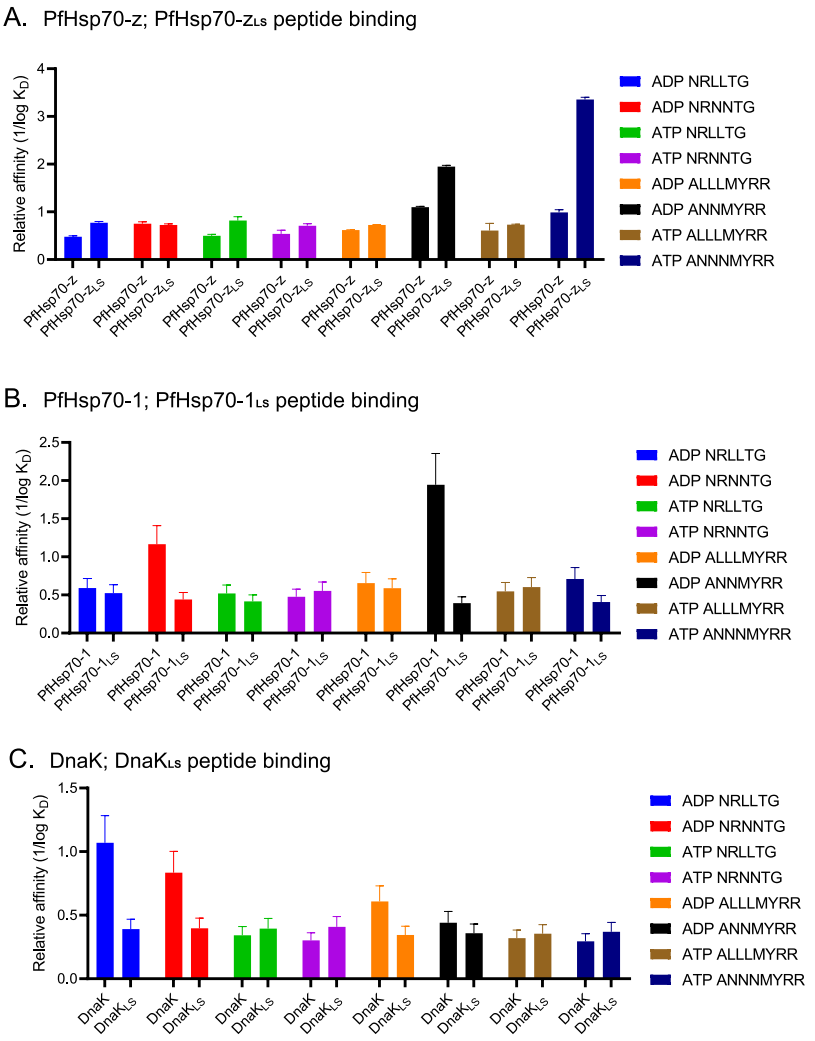

Fig. 4. Comparative effects of linker switch on the substrate binding properties of canonical Hsp70s and PfHsp70-z. The affinities of wild type Hsp70s and their linker mutants for various peptides were determined through SPR analysis. The bar graphs represent the peptide binding affinities (-log K_D) of PfHsp70-1/PfHsp70-1_{LS} (A), PfHsp70-z/PfHsp70-z_{LS} (B), and DnaK/DnaK$_{LS}$. (C). The data represent three independent repeat assays.

ATP in comparison to the ADP (Fig. 4A; Table S4). The findings suggest that the incorporation the linker of canonical Hsp70 conferred PfHsp70-z_{LS} with improved allosteric function.

As expected, in the presence of ADP both canonical Hsp70s (DnaK and PfHsp70-1) demonstrated increased affinity for the peptides (Fig. 4B and C; Table S4). ADP-induced affinity ranged from approximately 10-fold–100-fold for all the four peptides in comparison to the ATP-state of the chaperone (Fig. 4B and C; Table S4). This aligns with previous observations that highlighted that the chaperone activities of both PfHsp70-1 and DnaK are highly sensitive to nucleotide [17,31,32]. Notably, with the exception of NRLLTG, PfHsp70-1 exhibited higher affinity for the rest of the peptides than DnaK. In general, the linker mutants of both PfHsp70-1 and DnaK displayed reduced affinity for the peptides than their wild type forms. Altogether, the findings demonstrated that PfHsp70-1 is a more effective substrate binder than DnaK.

2.5. *The linker of Hsp70 regulates cellular client recognition*

DnaK is known to interact with approximately 700 proteins of which an estimated 180 are aggregation-prone [33]. Using DnaK and

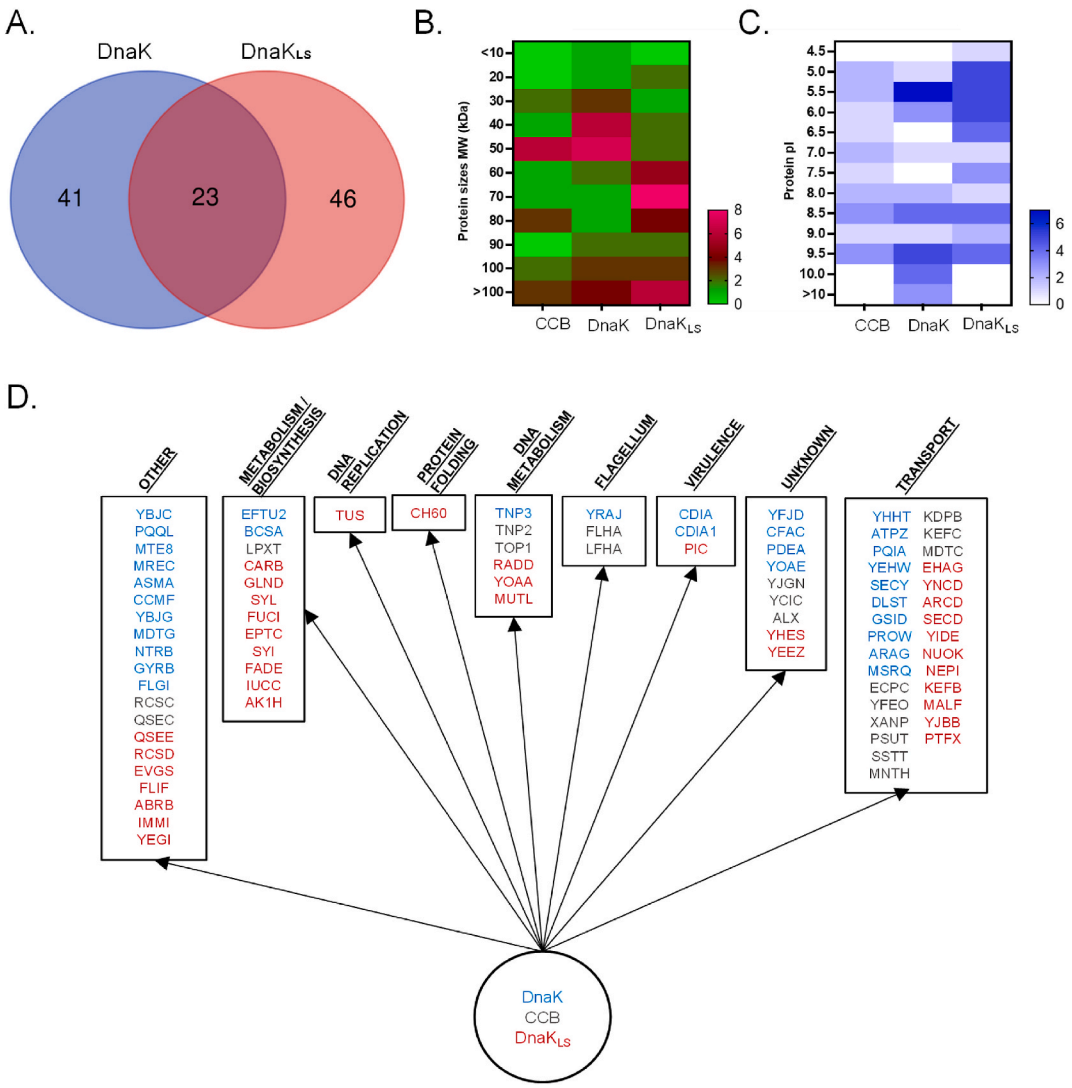

Fig. 5. Clients of DnaK and DnaK$_{LS}$ identified in *E. coli* JM109 cells. A co-affinity assay linked to LC-MS was used to establish clients of DnaK and DnaK$_{LS}$. A Venn diagram was used to illustrate the distribution of the clients (A). The clients of the two chaperones were further illustrated according to their sizes (B); pI (C) and pathways to which they belong (D). Clients common to both (CCB) chaperones are also illustrated. The raw data of client proteins is provided in Tables S5-S7.

its linker switch mutant as models, we investigated the role of the linker in modulating cellular client recognition using co-affinity approach coupled to LC-MS (Figure S5; Tables S5-S10). Firstly, we heterologously expressed polyhistidine tagged DnaK in two *E. coli* strains: JM109 cells (Promega, USA) and *E. coli* Δ*dnaK52* [BB1553 (MC4100 DdnaK52CmRsidB1)]. The *dnaK* gene of *E. coli* Δ*dnaK52* is intercepted by a *cat* cassette, which accounts for the thermosensitivity of this strain [34]. On the other hand, *E. coli* JM109 cells express a functional native DnaK protein.

We observed that 23 clients from *E. coli* JM109 cells co-eluted with both DnaK and $DnaK_{LS}$ (Fig. 5A; Tables S5-S7). DnaK co-eluted with an additional 41 clients from *E. coli* JM109 cells that were unique relative to the clients of $DnaK_{LS}$. Surprisingly, $DnaK_{LS}$ bound to 46 unique clients from *E. coli* JM109 cells. The association of $DnaK_{LS}$ with several unique clients confirms the data obtained by the *in vitro* peptide binding assays which point to the linker's role in regulating Hsp70 client selectivity. In this regard, the linker switch led $DnaK_{LS}$ to become less discriminate with respect to client selectivity. It is possible that the promiscuous client binding exhibited by

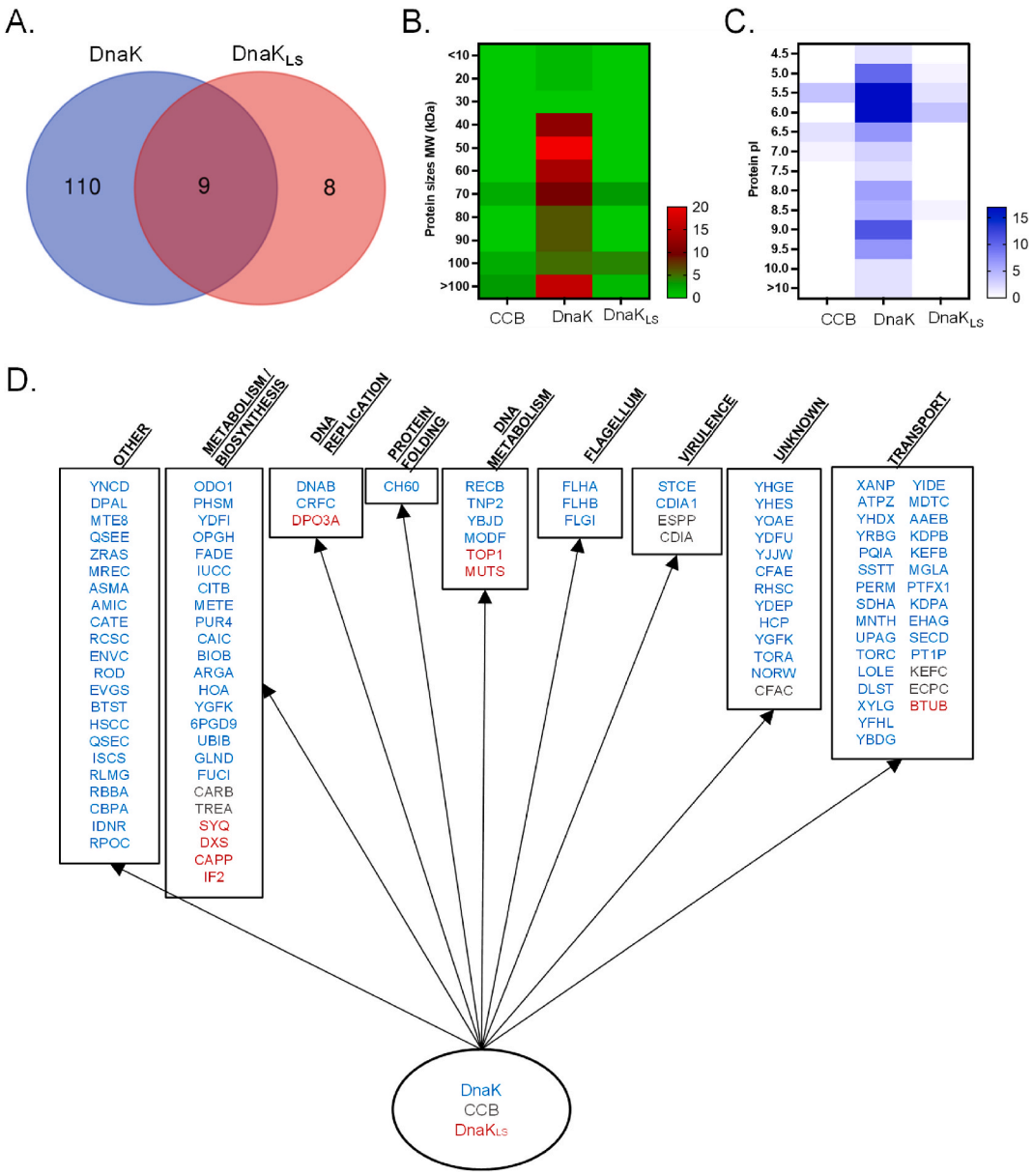

Fig. 6. Clients of DnaK and $DnaK_{LS}$ mapped from *E. coli ΔdnaK52* cells. Clients of DnaK and $DnaK_{LS}$ are shown in various scopes such as: Venn diagram (A); the heat map of sizes in kDa (B); their respective pI values (C) and the representative pathways to which they belong (D). For raw data of client proteins obtained refer to Tables S8-S10.

$DnaK_{LS}$ may not represent productive outcomes. Terminus utilization substance (TUS) and GroEL/chaperonin 60 (CH60) represent two unique clients of $DnaK_{LS}$. GroEL is a chaperone that is known to cooperate with DnaK in protein folding. However, there is no evidence suggesting that GroEL and DnaK directly interact although it has been suggested that the two chaperones may occur in ternary complexes by binding to a common substrate such as sigma 32 [35]. We also observed that the clients of DnaK and $DnaK_{LS}$ varied in sizes (Fig. 5B). Most of the clients that co-eluted with $DnaK_{LS}$ were larger than 70 kDa, while the majority of DnaK clients were around 50 kDa. Furthermore, analysis of the isoelectric points (pIs)of the clients (Fig. 5, panel C) showed that the majority of the DnaK clientele had pIs within 5.5–6.0 while some had pIs around 9.5. On the other hand, most of the clients of $DnaK_{LS}$ had pIs clustering around 5.0–6.5 while another group had pIs around 8.5–9.5. In addition, a few select clients of $DnaK_{LS}$ had pIs around 5.0, 6.5, 7.5 and 9.0. On the other hand, some of the clients unique of DnaK had pIs above 10. Altogether, the findings suggest that $DnaK_{LS}$ recruited more clients than DnaK and that the clients of the wild type DnaK generally exhibited varied features from those of its mutant.

In a follow up study, we further investigated the role of the linker in modulating DnaK clients in *E. coli* Δ*dnaK52* cells [31]. These cells lack a functional native DnaK as such, we sought to investigate the client selectivity of the heterologously expressed DnaK and $DnaK_{LS}$ in this setting. Notably, both DnaK and its mutant interacted with a total of 110 clients present in *E. coli* JM109 cells (Fig. 5, panel A; Table S5-S7). On the other hand, the two chaperones associated with 127 clients from *E. coli* Δ*dnaK52* cells. Notably, while $DnaK_{LS}$ interacted with 46 unique clients from *E. coli* JM109 (Fig. 5A), the same chaperone only interacted with 8 unique clients from *E. coli* Δ*dnaK52* cells (Fig. 6A; Tables S8-S10). On the other hand, DnaK bound to 110 clients from *E. coli* Δ*dnaK52* cells. The findings suggest that $DnaK_{LS}$ heterologously produced in *E. coli* Δ*dnaK52* cells was possibly misfolded and hence was unable to recruit clients effectively. Most of the clients of DnaK from *E. coli ΔdnaK52* cells were more spread in sizes from 40 to 75 kDa while another prominent cluster had sizes above 100 kDa (Fig. 6B). This demonstrates that the clients of DnaK from *E. coli ΔdnaK52* cells covered a wider size spectrum than those it bound to in *E. coli* JM109 cells. In addition, the clients of DnaK from *E. coli* Δ*dnaK52* cells had a wider pI range (Fig. 6, panel C) than those obtained from *E. coli* JM109 cells (Fig. 5, panel C). This further demonstrates the co-affinity assay conducted with DnaK recovered more misfolded proteins covering a vast spread of size and pI range than were recovered from *E. coli* JM109 cells which possessed a functional native DnaK. Notably, CH60/GroEL from *E. coli* JM109 cells was associated with $DnaK_{LS}$ and not with DnaK (Fig. 5, panel D). On the other hand, the same molecule was associated with DnaK within the *E. coli ΔdnaK52* cell background (Fig. 6, panel D). This suggests that DnaK cooperates more closely with GroEL in scenario representing a proteome undergoing an upheaval of stress as is synonymous with *E. coli* Δ*dnaK52* cells.

3. Discussion

This study reports for the first time, a systematic comparison of the roles of the linkers of canonical Hsp70s (represented by DnaK and PfHsp70-1) and Hsp110 (represented by PfHsp70-z) in regulating self-association and co-operation of these chaperones with their clients. Oligomerisation of Hsp70s is an important regulatory mechanism by which the availability of active monomeric forms of the chaperone is controlled in cells [5]. While some studies have explored oligomerisation of canonical Hsp70s [11,13,17], to be the best of our knowledge there are no reports on the oligomerisation of Hsp110 chaperones. Our findings demonstrated that the Hsp110 chaperone, PfHsp70-z, has strong propensity to self-associate (K_D of 1.39 nM in *apo*-state). Furthermore, its self-association was nucleotide independent which is synonymous with its behaviour as a nucleotide independent holdase chaperone [3]. Its linker mutant, $PfHsp70\text{-}z_{LS}$, exhibited lower but comparable self-association affinity (K_D, 2.75 nM in *apo*-state). This shows that although the linker of Hsp110 is important for the self-association of the chaperone, the mutation of this motif does not significantly lower its oligomerisation capability in *apo*- and ADP-states (Fig. 1; Table 1). However, in the presence of ATP, the self-association of the linker switch mutant of PfHsp70-z occurred with significantly weaker affinity. This highlights that the self-association of Hsp110 was sensitive to ATP upon the substitution of its linker with that of canonical Hsp70. One of the defining features of the linker of canonical Hsp70 is that it is highly flexible compared to the rigid linker of Hsp110 [10]. In this regard, the substitution of the linker of PfHsp70-z with that of canonical Hsp70s may confer nucleotide sensitivity which is crucial for oligomerisation. This is further supported by the fact that both ADP and ATP enhanced the heterologous association between $PfHsp70\text{-}z_{LS}$ and PfHsp70-1 in comparison to the *apo*-state (Fig. 2, panel B). Interestingly, PfHsp70-z's association with $PfHsp70\text{-}1_{LS}$ was not nucleotide sensitive (Fig. 2, panel C). This points that to the input of the rigid linker of PfHsp70-z present in $PfHsp70\text{-}1_{LS}$ which made the mutant protein insensitive to nucleotide modulation.

It has previously been reported that ATP promotes the self-association of canonical Hsp70s [17,36]. In agreement with this, the current study demonstrated that the self-association of both canonical Hsp70s (represented by PfHsp70-1 and DnaK) was promoted by ATP (Fig. 1; Table 1). The heterologous association between PfHsp70-1 and its linker switch mutant, $PfHsp70\text{-}1_{LS}$, occurred within comparable ranges of affinity as the oligomerisation of the wild type protein. However, the self-association of $PfHsp70\text{-}1_{LS}$ occurred at K_D values within the millimolar range, demonstrating that both linkers are a requirement for the self-association of canonical Hsp70s. We also observed that the PfHsp70-1 linker plays a more important role in self-association in comparison to that of DnaK (Table 1; Fig. 1). Altogether, our findings indicate that the highly conserved linker of canonical Hsp70s exerts a more pivotal role in driving self-association of these chaperones than the linker of their Hsp110 counterparts. We also speculate that, in addition to the linker, the self-association of Hsp70 is regulated by other segments of the chaperones, such as the NBD. A study by Aprile et al. [18] showed that

the oligomerisation propensity for the human Hsp70 NBD was significantly diminished (by more than 90 %) when it was not attached to the SBD, underscoring the cooperative roles of the NBD, linker and the SBD in oligomerisation of canonical Hsp70s. It has further been proposed that Hsp70 forms oligomers through SBD:SBD-; SBD:NBD-; and/or SBD:linker-based interactions [18]. These scenarios when effected in unique Hsp70 of various species origin would contribute in part to the specialised regulation of these chaperones. A previous study suggested that interactions between leucine residues in the SBD of one Hsp70 and leucine residues located in the linker of another Hsp70 mediate SBD:linker interactions during oligomer formation [18]. This may also explain why linker mutations modulate Hsp70 self-association.

Next, we explored the effect of the linker switch on the capability of the chaperones to bind to their functional partners *in vitro*. PfHsp70-z directly interacts with PfHsp70-1 possibly to drive nucleotide exchange function of the latter [3]. While ATP enhances the association of PfHsp70-z and PfHsp70-1 [3] (Fig. 2), the affinities for the interaction of either one of the linker mutants with wild type PfHsp70-1/PfHsp70-z or between both linker mutants were comparable in the presence of either ADP or ATP (Fig. 2; Table S2). We previously demonstrated that the interaction between PfHsp70-1 and PfHsp70-z occurs via their NBDs [3]. Thus, any changes that abrogate the sensitivity of the proteins to nucleotide adversely impact the association of the two proteins.

We further investigated the role of the linker of PfHsp70-1 in modulating its direct interaction with the co-chaperone, PfHop. PfHsp70-1 is known to interact with PfHop via its strongly electronegative C-terminal EEVD motif which recognises two (TPR1 and TPR2B) of the three electropositive domains of PfHop [22]. We previously demonstrated that PfHsp70-1_{LS} exhibits a unique tertiary structural conformation from its wild type form [10]. We propose that the conformation of PfHsp70-1_{LS} does not endear to PfHop binding. In addition, the observed weak affinity of PfHop for PfHsp70-1_{LS} was nucleotide independent. We previously employed intrinsic fluorescence to demonstrate that PfHsp70-1_{LS} exhibits poor conformational flexibility [10]. It is thus plausible that, the binding of nucleotide at the NBD does not induce typical interdomain coupling crucial for efficient PfHop co-chaperone binding by the linker switch mutant of PfHsp70-1.

Although PfHsp70-1 and PfHsp70-z linker mutations were previously demonstrated to adversely impact peptide substrate recognition, the previous study relied on using a single peptide and its Asp- rich derivative [10]. In the current study, we expanded the study by including two more peptides together with their Asp-rich forms. Findings from the current study confirm that the linkers of the canonical Hsp70s and Hsp110 are involved in regulating substrate binding affinity (Fig. 3). Previous studies have indicated that minor SBDβ alterations can induce distinct allosteric coupling effects by controlling the movement of the α-helical lid and linker segments [37]. *In silico* analyses indicated that swapping the linkers of PfHsp70-z and PfHsp70-1 led to structural variations within the SBDβ and SBDα regions of the proteins (Figure S1-S3). This alteration potentially affected the substrate binding preferences of the

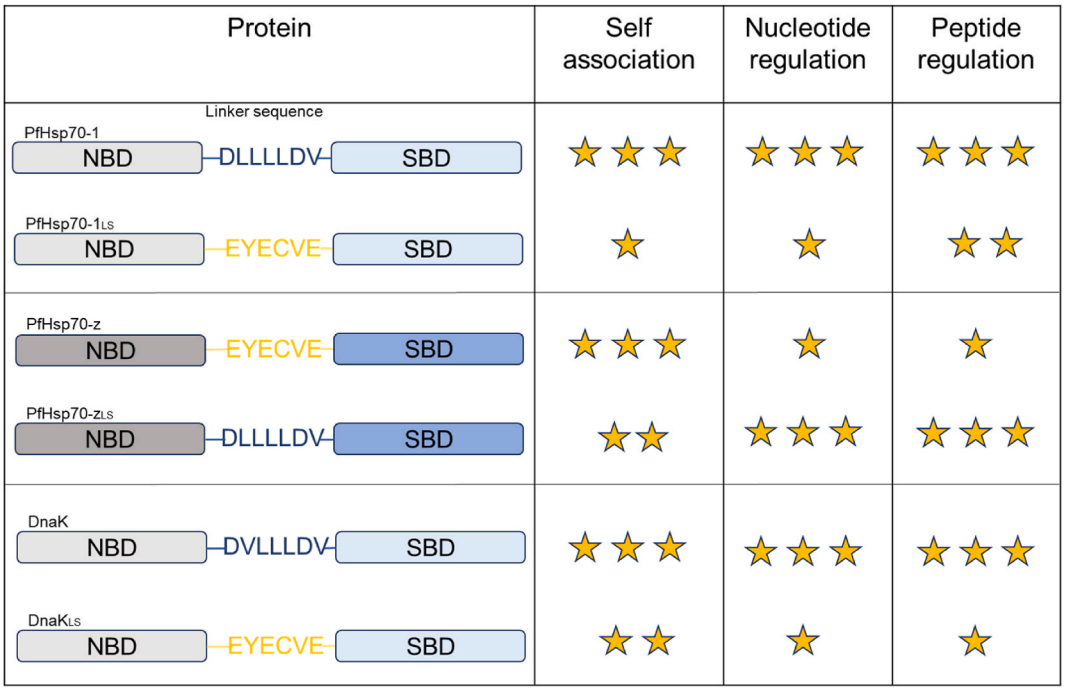

Fig. 7. Summary of the effects of linker swaps on the functions of PfHsp70–1, DnaK and PfHsp70-z. Schematic representation of the Hsp70s and their chimeric linker mutants. The linker sequences are shown. The effects of the linker swaps on the various functional features such as self-association, regulation by nucleotide and peptide recognition are depicted by star symbols to represent their respective levels of functional sensitivity.

proteins. Although PfHsp70-z demonstrated a greater affinity for Asp-enriched peptide substrates, PfHsp70-z_{LS} bound both the original and Asp-enriched peptides with similar affinities (Fig. 4; Table S4). This observation further supports that PfHsp70-z prefers Asp-enriched substrates, as previously reported for another *P. falciparum* Hsp70 (PfHsp70-x) [24]. Swapping the linker of PfHsp70-z with that of PfHsp70-1, possibly improved the flexibility of PfHsp70-z_{LS}, enabling the protein to adopt a conformation that enhanced peptide binding.

Using co-affinity approach coupled to LC-MS, we mapped out the possible cellular clients of DnaK relative to those of its mutant, $DnaK_{LS}$. Our findings demonstrated that DnaK and its mutant bound to more clients from *E. coli* JM109 than from *E. coli* Δ*dnaK52* cells. The reduced clientele of the chaperones within the *E. coli* Δ*dnaK52* system suggests that the lack of functional DnaK in these cells may have reduced the population and quantum of the proteomic constituents as compared to *E. coli* JM109 cells which contains a functional native DnaK. These findings suggest that the absence of DnaK broadly reduces the cellular proteome. Furthermore, we demonstrated that DnaK is capable of binding to several cellular clients spanning various sizes and pI ranges. We further established that the diversity of DnaK clients cover broader scope with respect to population, pI range, and size in *E. coli* Δ*dnaK52* cells than in *E. coli* JM109 cells. This shows that the absence of a native DnaK in *E. coli* Δ*dnaK52* adversely impacts the development of this strain. That DnaK was able to recognise such a wider scope of clients from *E. coli* Δ*dnaK52* cells demonstrates the functional elasticity of this chaperone. Indeed, DnaK is known to account for folding of not less than 700 clients of which 180 are unstable [33]. This vast array of clients, some of whom are aggregation prone is naturally expanded in *E. coli* strains whose protein quality control is weak such as *E. coli* Δ*dnaK52* cells. The absence of a native DnaK in *E. coli* Δ*dnaK52* cells is reflected by the fact that $DnaK_{LS}$ bound only to 17 unique residues from this strain compared to 69 clients from *E. coli* JM109 cells that were recognised by the same mutant chaperone. The poor recovery of clients from *E. coli* Δ*dnaK52* cells by $DnaK_{LS}$ is possibly due to the reduced proteomic composition of the cells due to lack of DnaK function. Not only did *E. coli* Δ*dnaK52* cells produce a reduced proteomic complement but it is conceivable that several of its proteins occurred in aggregated form. Thus, in comparison to DnaK, $DnaK_{LS}$ bound to fewer clients from *E. coli* Δ*dnaK52* cells. Furthermore, the clients covered broader size and pI ranges than those bound by DnaK. This shows that $DnaK_{LS}$ demonstrated reduced cellular client selectivity. Thus, our findings highlight the linker of Hsp70 as an important determinant of substrate binding. In line with this, we recently observed that insertion of a GGMP repeat segment of PfHsp70-1 in the C-terminal lid of DnaK compromised the functional stability and substrate selectivity of the latter [38]. In conclusion, our findings demonstrated that the linker is important for oligomerisation of Hsp70. In addition, we established that this motif is more important for the self-association of canonical Hsp70 than their Hsp110 counterparts (Fig. 7). We further established that although this motif is particularly important in the self-association of canonical Hsp70s, its influence varies across the chaperones suggesting that other segments such as the NBD and SBD make contributions to the organisation of Hsp70 oligomers. Finally, we demonstrated an important role for the linker in regulating Hsp70 client's selection (Fig. 7). While the linker is highly conserved in canonical Hsp70s, it is more divergent in non-canonical Hsp70s (Hsp110 an Grp170) [5,10]. For this reason, it is important to conduct further studies to establish the unique roles of the linkers present in various Hsp110 members.

4. Limitations of study

While SPR analysis allowed us to establish self-association of the Hsp70 chaperones, we could not infer on the levels of oligomerisation of the respective proteins. In addition, due to lack of access to a *Plasmodium falciparum* cell culture facility we restricted the study on cellular client selection to *E. coli* as a model.

5. Methods

5.1. Recombinant protein expression and purification

Linker motif swap (LS) variants of PfHsp70-1, PfHsp70-z and DnaK, denoted as PfHsp70-1_{LS}, PfHsp70-z_{LS} and $DnaK_{LS}$, respectively, were created by substitution mutations of the respective linkers as previously described [10]. Expression and purification of the Hsp70s (PfHsp70-1, PfHsp70-z, PfHsp70-1_{LS}, PfHsp70-z_{LS}, DnaK, $DnaK_{LS}$) was conducted as previously described [10].

5.2. SPR analysis to explore effects of linker mutations on the Hsp70 self-association and interaction with functional partners

To determine the self-interaction kinetics of Hsp70s and their LS variants, multi parametric surface plasmon resonance (MP-SPR) was performed using the BioNavis MP-SPR Navi 420A ILVES system at 25 °C as previously described [10]. This technique was also used to assess PfHsp70-1's direct association with its functional partner PfHop. Following immobilization of ligands (PfHsp70-1/z/Hop) onto CMD sensor chips, the analytes (PfHsp70-1, PfHsp70-1_{LS}, PfHsp70-z, PfHsp70-z_{LS}, DnaK and $DnaK_{LS}$, PfHop) were injected in

series as aliquots of 0, 125, 250, 500, 1000, and 2000 nM and a flow rate of 50 μl/min into each flow channel as previously described [10]. This was followed by equilibrium affinity analysis. Statistical analyses were conducted using a One-Way ANOVA at a 99 % confidence interval ($p < 0.01$).

5.3. Determination of effect of Hsp70 linker switch on peptide binding

The binding affinities of PfHsp70-1_{LS}, PfHsp70-z_{LS}, $DnaK_{LS}$ and their respective wild type forms for model peptide substrates NRLLTG, NRNNTG, ALLLMYRR and ANNNMYRR were determined by SPR as previously reported [10]. Steady-state equilibrium kinetics were processed and analysed using TraceDrawer software version 1.8 (Ridgeview Instruments, Sweden).

5.4. Analysis of the effects of the linker mutation on the interaction of DnaK with cellular clients

A co-affinity assay coupled to downstream LC-MS analyses were used to investigate the effect of linker mutations on the DnaK interactome as previously described [3]. Purified recombinant DnaK and $DnaK_{LS}$ were immobilized (as bait) by His-tag affinity on HisPur cobalt resin suspended in TBS (10 mM Tris-HCl pH 7.5, 50 mM NaCl and 10 Mm imidazole) and incubated at 4 °C for 4 h with gently rocking motion. To remove the unbound ligand, the cobalt resin was washed with 5-bed volumes of wash buffer (1:1 TBS: Pierce lysis buffer™) and centrifuged at 1250×*g* for 1 min. The wash steps were repeated 5 times. *E. coli Δdnak52* and *E. coli* JM109 cell lysates were used as prey. The prey was incubated overnight with respective immobilized bait protein at 4 °C with gentle rocking. After incubation, unbound proteins were washed 5 times using a wash buffer. Bound prey-bait proteins were eluted from the HisPur cobalt resin using elution buffer (Pierce wash buffer supplemented with 290 mM imidazole). The co-affinity chromatograph was analysed using SDS-PAGE analysis. The SDS-PAGE gel was stained using Pierce silver stain for LC-MS (Thermo Scientific, USA) following manufacture's protocol. Unique protein bands were excised for analysis. LC-MS analyses were then conducted to identify the proteins that co-eluted with the DnaK and $DnaK_{LS}$, as previously described [39].Statistical analysis was performed by student t-test using GraphPad Prism version 10.0, GraphPad Software, San Diego, California USA, (www.graphpad.com).

Data availability statement

The raw proteomics data is included in supplementary section as cited in the manuscript. The raw data obtained by LCMS is available on the jPOST repository with ID: JPST002494[41].

CRediT authorship contribution statement

Graham Chakafana: Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Caitlin J. Middlemiss:** Methodology, Investigation. **Tawanda Zininga:** Writing – original draft, Supervision. **Addmore Shonhai:** Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix ASupplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29690.

Appendix

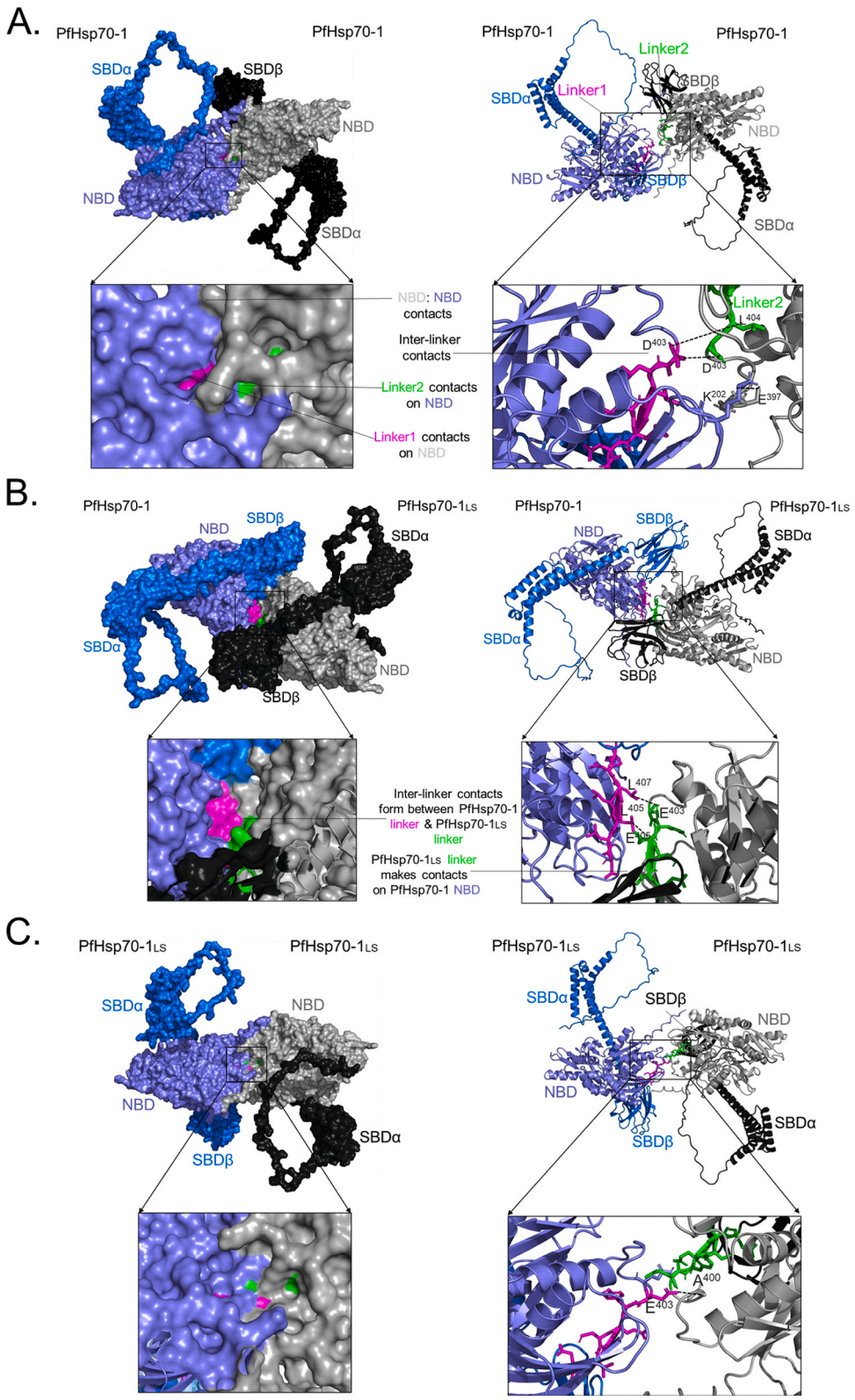

Fig. S1**Three dimensional models depicting the self-association of PfHsp70–1.** Representative three-dimensional models generated by HADDOCK show that (A) the linkers of PfHsp70-1 monomers make direct contacts as well as those located in the NBD of the adjacent monomer

during self-association. (B) Similarly, the association of PfHsp70-1 and PfHsp70-1_{LS} is predicted to be driven through linker contacts with the NBD and SBD segments. (C) PfHsp70-1_{LS} self-association is predicted to occur through unique linker NBD contacts facilitated by the linker residue, E^{403}.

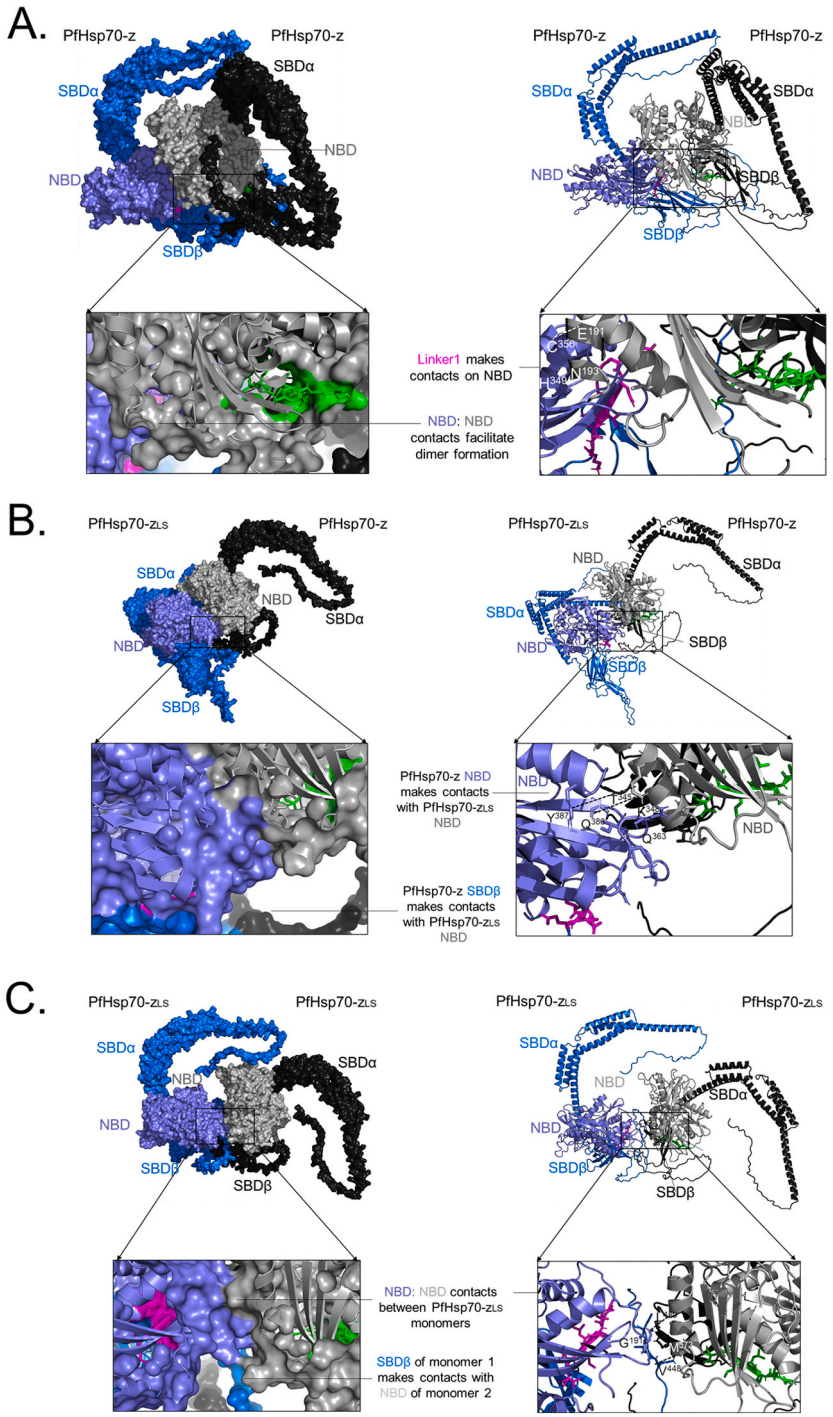

Fig. S2. Three dimensional models of PfHsp70-z demonstrating how the linker switch impacts self-association. Representative three-dimensional model generated by HADDOCK show that (A) PfHsp70-z self-association occurs through linker:NBD, NBD:NBD and NBD:SBD contacts. (B) The association of PfHsp70-z and PfHsp70-z_{LS} is also predicted to predominantly occur through NBD:NBD contacts. (C) Similarly, PfHsp70z-$_{LS}$ self-association is predicted to occur through NBD:NBD and NBD:SBD contacts between the monomers.

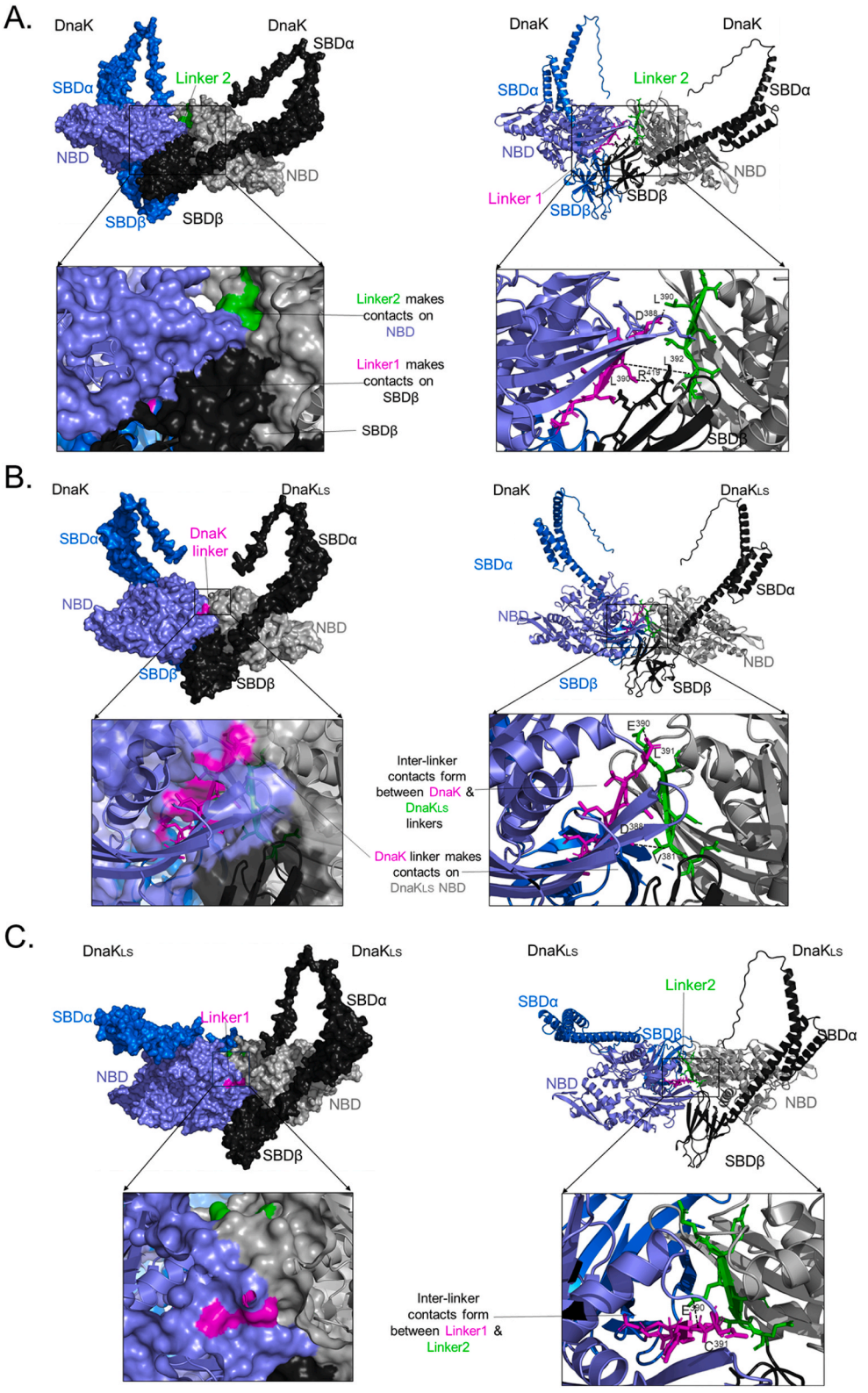

Fig. S3. Three dimensional models of DnaK demonstrating effect of the linker switch on its self-association. Representative three-dimensional models generated by HADDOCK show that (A) DnaK self-association occurs through linker: NBD, NBD:NBD and NBD:SBD contacts between individual monomers; (B) the association of DnaK and $DnaK_{LS}$ is predicted to occur through unique linker: linker and linker: NBD contacts

between residues of the individual monomers; and (C) DnaK$_{LS}$ self-association is predicted to occur through a single linker: linker contact and NBD contacts between monomers.

Table S1
Linker residues predicted to modulate the self-association of the Hsp70s and their derivatives

Monomer 1	Monomer 2	Contact residues
PfHsp70–1	PfHsp70-1	**D**^{403}; **L**^{404} **D**^{403}; **D**^{403}
PfHsp70-1$_{LS}$	PfHsp70-1	**L**^{405}; **E**^{405} **L**^{404}; **E**^{405} **L**^{407}; **E**^{403}
PfHsp70-1$_{LS}$	PfHsp70-1$_{LS}$	**E**^{403}; A^{400} Q^{402}; **E**^{403}
DnaK	DnaK	**D**^{388}; **L**^{390} **L**^{390}; R^{419} **L**^{390}; **L**^{392} K^{421}; **L**^{392}
DnaK$_{LS}$	DnaK	**L**^{391}; **E**^{390} **D**^{388}; V^{381}
DnaK$_{LS}$	DnaK$_{LS}$	**C**^{391}; **E**^{390}
PfHsp70-z	PfHsp70-z	– –
PfHsp70-z$_{LS}$	PfHsp70-z	– –
PfHsp70-z$_{LS}$	PfHsp70-z$_{LS}$	– –

*Linker residues are highlighted in bold. Note, no linker residues are predicted to be implicated in the following dimer pairs: PfHsp70z:PfHsp70-z; PfHsp70-z$_{LS}$:PfHsp70-z; PfHSsp70-z$_{LS}$:PfHsp70-z$_{LS}$.

Table S2
SPR data for the self-association of the Hsp70s and their linker switch derivatives

Ligand	Analyte	Ka (1/Ms)	Kd (1/s)	K_D (M)	χ [2]
PfHsp70-1	PfHsp70-1 (NN)	2.36 (±0.02)e [4]	8.83 (±0.02)e^{-2}	3.75 (±0.05)e^{-6}***	0.08
	PfHsp70-1 (ADP)	8.47 (±0.05)e [3]	1.09 (±0.05)e^{-2}	1.29 (±0.09)e^{-6}	8.98
	PfHsp70-1 (ATP)	7.14 (±0.03)e [4]	1.91 (±0.06)e^{-2}	2.68 (±0.08)e^{-7}	7.67
PfHsp70-1$_{LS}$	PfHsp70-1 (NN)	1.99 (±0.09)e [4]	9.35 (±0.05)e^{-2}	4.70 (±0.70)e^{-6}	0.76
	PfHsp70-1 (ADP)	1.60 (±0.02)e [4]	5.39 (±0.04)e− [2]	3.37 (±0.07)e^{-6}	7.29
	PfHsp70-1 (ATP)	3.95 (±0.04)e [4]	3.92 (±0.06)e^{-2}	9.98 (±0.08)e^{-7}	3.82
PfHsp70-1$_{LS}$	PfHsp70-1$_{LS}$ (NN)	1.78 (±0.05)e [3]	4.47 (±0.08)e^{-2}	4.24 (±0.04)e^{-5}***	0.64
	PfHsp70-1$_{LS}$ (ADP)	3.67 (±0.07)e [3]	6.33 (±0.09)e^{-2}	1.72 (±0.02)e^{-5}	4.94
	PfHsp70-1$_{LS}$ (ATP)	1.13 (±0.08)e [3]	6.66 (±0.02)e^{-2}	5.90 (±0.90)e^{-5}	3.93
DnaK	DnaK (NN)	2.72 (±0.02)e [4]	6.21 (±0.01)e^{-2}	2.28 (±0.08)e^{-6}***	0.07
	DnaK (ADP)	4.38 (±0.08)e [4]	8.19 (±0.09)e^{-2}	1.89 (±0.09)e^{-6}	0.01
	DnaK (ATP)	6.33 (±0.03)e [4]	3.19 (±0.09)e^{-2}	5.04 (±0.04)e^{-7}	2.19
DnaK$_{LS}$	DnaK (NN)	4.57 (±0.07)e [4]	9.21 (±0.01)e^{-2}	2.02 (±0.02)e^{-6}	5.76
	DnaK (ADP)	7.32 (±0.02)e [4]	8.26 (±0.06)e^{-2}	1.13 (±0.03)e^{-6}	1.98
	DnaK (ATP)	6.32 (±0.02)e [4]	5.71 (±0.01)e− [3]	9.03 (±0.03)e^{-8}	0.09
DnaK$_{LS}$	DnaK$_{LS}$ (NN)	2.39 (±0.09)e [3]	7.14 (±0.04)e^{-2}	2.97 (±0.07)e^{-5}***	1.78
	DnaK$_{LS}$ (ADP)	4.39 (±0.09)e [3]	9.03 (±0.03)e^{-2}	2.05 (±0.05)e^{-6}	8.98
	DnaK$_{LS}$ (ATP)	9.06 (±0.06)e [3]	4.92 (±0.02)$^{-2}$	5.40 (±0.40)e^{-6}	2.76
PfHsp70-z	PfHsp70-z (NN)	3.79 (±0.09)e [4]	5.27 (±0.07)e^{-5}	1.39 (±0.09)e^{-9}	8.47
	PfHsp70-z (ATP)	8.93 (±0.03)e [3]	7.73 (±0.03)e^{-5}	9.08 (±0.08)e^{-9}	6.74
	PfHsp70-z (ADP)	1.15 (±0.05)e [4]	3.37 (±0.07)e^{-5}	2.93 (±0.03)e^{-9}	5.42
PfHsp70-z$_{LS}$	PfHsp70-z (NN)	1.31 (±0.01)e [4]	4.15 (±0.05)e^{-5}	3.17 (±0.07)e^{-9}	2.17
	PfHsp70-z (ATP)	2.37 (±0.07)e [4]	4.36 (±0.06)e− [5]	1.84 (±0.04)e^{-9}	0.19
	PfHsp70-z (ADP)	1.91 (±0.01)e [4]	5.32 (±0.02)e^{-5}	2.79 (±0.09)e^{-9}	0.87
PfHsp70-z$_{LS}$	PfHsp70-z$_{LS}$ (NN)	1.59 (±0.09)e [4]	4.37 (±0.07)e^{-5}	2.75 (±0.05)e^{-9}	1.02
	PfHsp70-z$_{LS}$ (ATP)	2.11 (±0.01)e [4]	3.99 (±0.09)e^{-4}	1.89 (±0.09)e^{-8}	3.45
	PfHsp70-z$_{LS}$ (ADP)	4.11 (±0.01)e [4]	3.97 (±0.07)e^{-4}	9.66 (±0.06)e^{-9}	2.97

The data represents three independent analyses. The statistical significance of the K_D value obtained for a respective assay relative to findings obtained for the association of wild type monomers of the respective Hsp70 in the absence of nucleotide (NN) was determined by a one way ANOVA test at $p < 0.01$***.

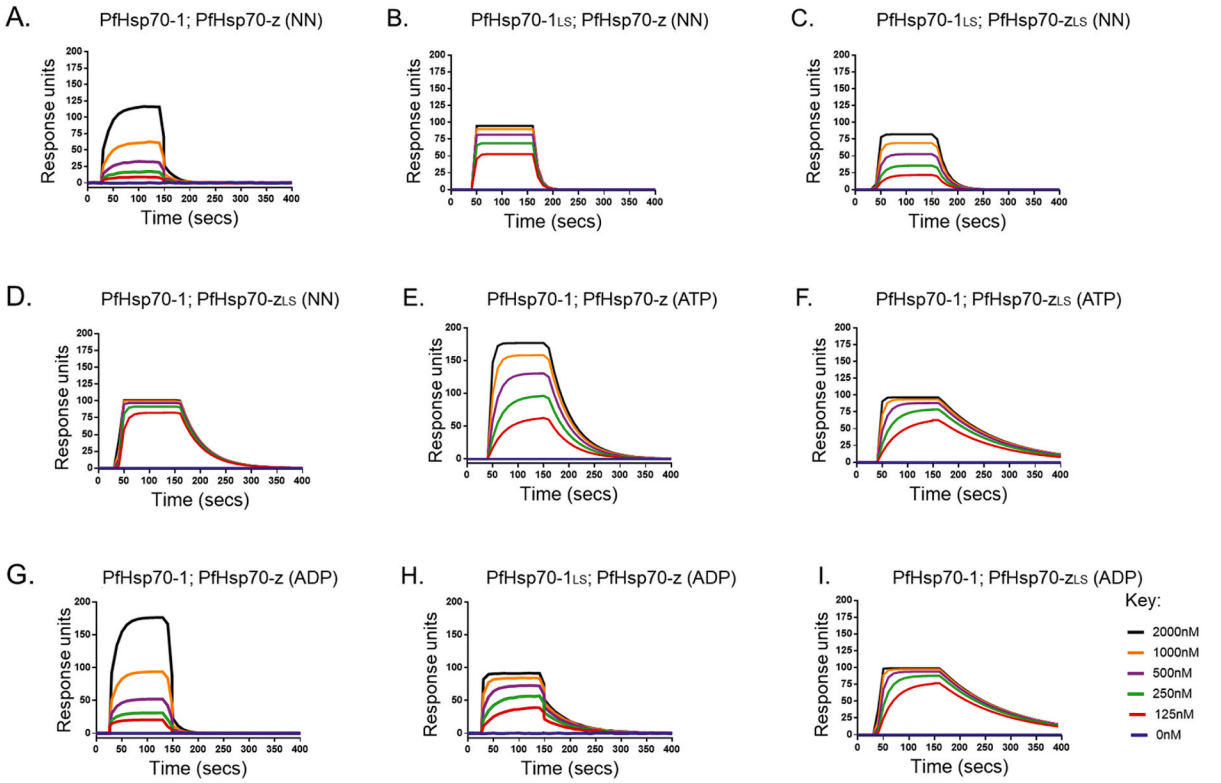

Fig. S4. SPR sensorgrams representing effects of linker substitutions on the association of PfHsp70-1 and PfHsp70-z. Interaction between various monomers was investigated: wild type PfHsp70-1:PfHsp70-z in the absence of nucleotides (A); PfHsp70-1_{LS}:PfHsp70-z in the absence of nucleotides (B); PfHsp70-z_{LS}:PfHsp70-1_{LS} in the absence of nucleotide (C); PfHsp70-1:PfHsp70-z_{LS} in the absence of nucleotide (D); PfHsp70-1: PfHsp70-z in the presence of ATP (E); PfHsp70-1:PfHsp70-z_{LS} in the presence of ATP (F); PfHsp70-1:PfHsp70-z in the presence of ADP (G); PfHsp70-1LS:PfHsp70-z in the presence of ADP (H); and PfHsp70-1:PfHsp70-z_{LS} in the presence of ADP (I). The assays were repeated in the presence of varying concentrations of analyte.

Table S3

SPR kinetics data for interaction of PfHsp70-1/PfHsp70-1_{LS} and PfHop

Ligand	Analyte	Ka (1/Ms)	Kd (1/s)	KD (M)	χ [2]
PfHsp70-1	PfHop (NN)	3.54e [3]	$1.38e^{-5}$	3.89 (±0.09) e^{-9}	1.62
	PfHop (ADP)	6.45e [4]	$4.99e^{-4}$	7.75 (±0.05) e^{-9}	1.26
	PfHop (ATP)	2.10e [5]	$1.99e^{-2}$	9.46 (±0.06) e^{-8}	2.43
PfHsp70-1_{LS}	PfHop (NN)	4.82e [5]	$1.34e^{-2}$	2.77 (±0.07) e^{-8}	2.31
	PfHop (ADP)	1.00e [5]	$1.00e^{-3}$	1.00 (±0.09) e^{-8}	2.07
	PfHop (ATP)	8.80e [5]	$2.35e^{-3}$	3.76 (±0.06) e^{-8}	1.89

Three independent assays to explore interaction of PfHop with PfHsp70-1/PfHsp70-1_{LS} were determined by SPR analysis. Standard errors are shown in brackets. Chi [2] values indicate the score for the goodness of fit of the model used to generate the kinetics.

Table S4

Comparative peptide binding affinities of Hsp70s and their linker switch variants

Protein	**Nucleotide**	K_D peptide binding affinity (nM)			
		NRLLTG	**NRNNTG**	**ALLLMYRR**	**ANNNMYRR**
PfHsp70–1	ATP	181 (±0.01)	296 (±0.07)	140 (±0.40)	45.4(±0.07)
	ADP	97.4 (±0.04)	10.2 (±0.02)	61.8 (±0.08)	4.02(±0.02)
PfHsp70-1_{LS}	ATP	682 (±0.02)	132 (±0.02)	89.4 (±0.04)	778(±0.08)
	ADP	174 (±0.04)	466 (±0.06)	99.3 (±0.03)	977(±0.07)
PfHsp70-z	ATP	128 (±0.08)	52.5 (±0.05)	97.1 (±0.09)	8.31(±0.01)
	ADP	145 (±0.03)	24.1 (±0.01)	43.1 (±0.01)	9.37(±0.07)
PfHsp70-z_{LS}	ATP	20.7 (±0.07)	59.5 (±0.07)	36.5 (±0.05)	2.00(±0.09)
	ADP	21.1 (±0.01)	25.6 (±0.06)	41.6 (±0.06)	3.30(±0.30)
DnaK	ATP	2530 (±30)	7310 (±10)	4450 (±50)	8960(±60)
	ADP	12.3 (±0.03)	24.9 (±0.09)	82.0 (±0.02)	440(±4.0)
DnaK$_{LS}$	ATP	891 (±1.0)	716 (±6.00)	1950 (±5.0)	1770(±7.0)
	ADP	962 (±5.00)	855 (±5.00)	2440 (±4.0)	1690(±9.0)

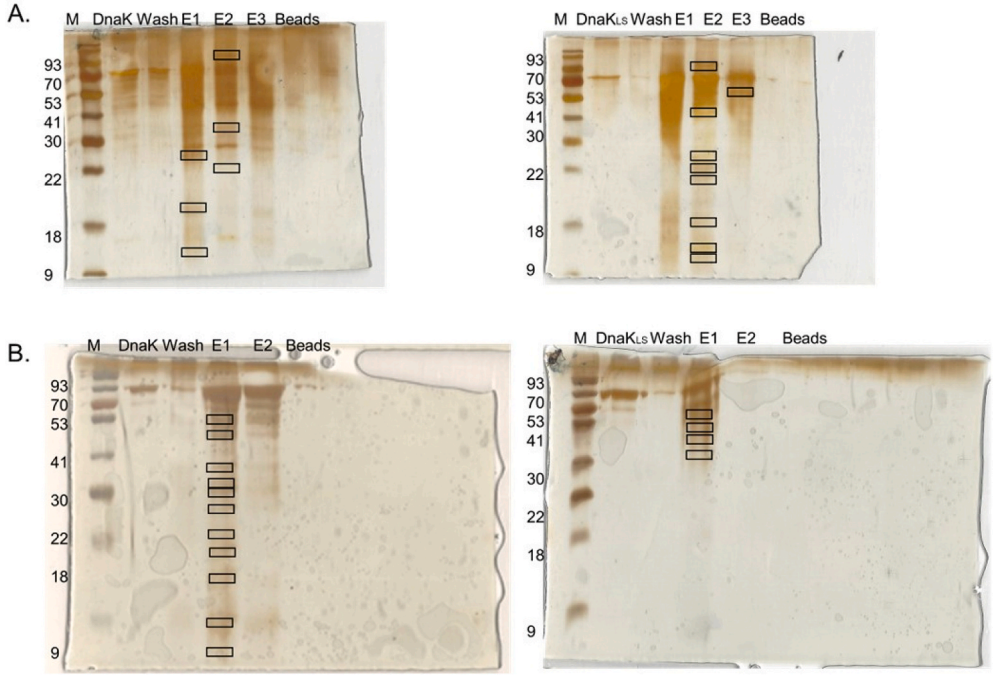

Fig. S5. Silver stained SDS-PAGE representing pull down assay constituents. The silver-stained gels shown were obtained following pull-down assays conducted using either DnaK/DnaK$_{LS}$ as bait with the cell lysates of: *E. coli* JM109 (A) and *E. coli ΔdnaK52;* (B) Lane ''M″ represents the molecular weight markers, DnaK/DnaK$_{LS}$ represents the respective bait protein attached to His-column, ''Wash'' represents the fractions collected after washing off non-specifically bound proteins. The bands highlighted in the black rectangles represent the unique bands that were excised from the gels and analysed by LC-MS. His-beads without immobilized bait protein served as control. E1-E2, represent elutions 1 and 2, respectively.

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