Supplemental Figure Legends

Supplemental Figure 1. Thermodynamic analysis of Tomm34 binding to Hsp70•ATP by ITC. (*A*) Raw ITC data showing Hsp70-Tomm34 interaction in the absence of ATP at indicated temperatures. (*B*) Control ITC experiments, where buffer was injected into Hsp70 (with ATP), Tomm34 was injected into buffer (with ATP) without protein or Tomm34 was injected into Hsp70 in the absence of ATP. All measurements were done at 15 °C. (*C*, *D*) Analysis of temperature dependence of Hsp70•ATP-Tomm34 interaction. At 25 °C, it was not possible to analyze thermodynamic parameters due to very small enthalpy change for the interaction. (*E*) Dissociation constant (*K*_d) of Hsp70•ATP-Tomm34 complex is plotted as a function of temperature. (*F*) Temperature dependence of enthalpic and entropic contribution to the Gibbs free energy of ATP-dependent Hsp70-Tomm34 interaction.

Supplemental Figure 2. Hsp70 T204A exhibits decreased ATPase activity in comparison to WT. Hop inhibits Hsp70/Hsp40-mediated refolding only slightly and at higher concentrations. (*A*) The ATPase activity of Hsp70 proteins (2 μ M) was tested at various Hsp40 concentrations in malachite green assay. (*B*) Firefly luciferase was chemically denatured, mixed with Hsp70 (1 μ M), Hsp40 (2 μ M), ATP (1 mM), and varying Hop concentrations, and then recovered luminescence was measured.

Supplemental Figure 3. The effect of I164D and D529A mutations, and interstitial deletion of 533-543 region on Hsp70 conformational activity and substrate binding. (*A*) Deuteration level differences of Hsp70 (I164D/D529A/ Δ 533-543) peptides in ATP-bound and nucleotidefree state after 1 h incubation in deuterated buffer. (*B*) Equilibrium binding curves of F-NRLLLTG peptide binding to Hsp70 WT and mutants under nucleotide-free conditions. Fluorescence polarization was determined at 30 nM peptide and increasing Hsp70 concentrations. Experiments were performed in independent triplicates. Error bars represent S.E. (*C*) Kinetics of F-NRLLLTG interaction with WT and mutant Hsp70s under nucleotide-free conditions. Protein and peptide concentrations were 25 μ M and 30 nM, respectively.

Supplemental Figure 4. The structure of Tomm34 is not affected by ATP. Deuteration level differences of Tomm34 peptides in the presence and absence of ATP (5 mM) after 10 min/3 h incubation in deuterated buffer.

Supplemental Figure 5. The Tomm34 interdomain linker contains stretch of evolutionary conserved residues predominantly of hydrophobic nature. (*A*) The prediction of Tomm34 secondary structure as performed by PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred). The interdomain linker (approximately 140-190, indicated with red line) separates TPR1 (yellow line) and TPR2 (green line) domains of Tomm34. (*B*) Multiple sequence alignment of Tomm34 protein sequences from various species (sequences obtained from Uniprot database, http://uniprot.org). The upper panel shows part of N terminal TPR1 domain. Blue box indicates the residue at the first position of two-carboxylate clamp. The canonical lysine (K) has mutated to arginine (R) in the course of evolution. The bottom panel depicts the alignment of interdomain linker sequences with the conserved hydrophobic region highlighted by the red box. The multiple sequence alignment was performed by DNASTAR Lasergene Suite (http://dnastar.com).







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| Pred: 2 () | 3 | 4 5 - 6 5 |
| Pred: CCCCCCCHHHHHHHHHHHHHHHCCCHHHHHHHHHHHH | HCCCCCCCCHHHHHHHHHHHHHHCCCCHHHHH AQGSSDPEEESVLYSNRAACHLKDGNCRDCIK | HHHHHHHC CCCCHHHHHHHHHHHHHCCCHHHHHHHHHH |
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| Pred: 7 () () | | 1 2 3 |
| Pred: HHHHHHHHHHHHHHHCCCCHHHHCCCCCCCCHHHHHHHCCC AA: AVEGINRMTRALMDSLGPEWRLKLPSIPLVPVSAQKRWNS | CCCCCHHHHHHCCCCHHHHHHCCCCCCCCCHHH LPSENHKEMAKSKSKETTATKNRVPSAGDVEK | HHHHHHHH ARVLKEEG NELVKKGNHKKAIEKYSESLLCSNLESATYSNRALCYLVL |
| 130 140 150 16 | 0 170 180 190 | 200 210 220 230 240 |
| Conf:]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]]] | <u>]]</u>]]]]]]]]]]]]]]]]]]]]]]]]]]]]]] | Legend: TPR1 linker TPR2 |
| Pred: 4 5 | 6 7 | - helix Conf:]]] [- confidence of prediction |
| Pred: CCHHHHHHHHHHHHHCCCCHHHHHHHHHHHHHHCCCHHHH | HHHHHHHHCCCCCHHHHHHHHHHHHHHCC FADISNLLQIEPRNGPAQKLRQEVKQNLH | - + - strand Pred: predicted secondary structure |
| 250 260 270 28 | 0 290 300 | = coil AA: target sequence |

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| sp Q15785 TOM34_HUMAN | SVEELRA | AGNESF | RNGQY | A E A S A L Y | GRALRVLQA | AQGSSDPE | EESVLYS | NRAACHL | K D G N C R D C I K |
|---|---|--|--|--|--|---|--|--|--|
| Sequence Ruler | 10 | 2 | 0 | 30 | 40 | | 50 | 60 | 70 |
| splQ9CYG7 TOM34_MOUSE | SVEQLRA | AGNQNF | RNGQY | GEASALY | ERALRLLQA | ARGSADPE | EESVLYS | NRAACYL | KDGNCTDCIK |
| tr E1BGD1 E1BGD1_BOVIN | SVEELR | T <mark>G</mark> NQ S F | R N G Q F | FAEAATLY | S <mark>R A L R</mark> M L Q E | EQGSSDPE | K E S V L Y S | NRAACHL | K DGNC I DC I K |
| tr F1SDR5 F1SDR5_PIG | SVEELRA | A G N Q S F | R N G Q F | FAEAATLY | S R A L R M L Q A | AQGSLDPE | K <mark>E</mark> S V L F S | NRAACHL | K DGNC V DC I K |
| tr F1P4X4 F1P4X4_CHICK | S A G D L R F | A G N E E F | R R GQ Y | GAAAELY | S R A L A V L E C | D A G E A A A E | ERSVLLA | NRAACQL | RDGACRGCVA |
| tr V8P625 V8P625_Cobra | - IADLKO | A GN EQ F | RNGQY | GQAAALY | GRALELLEA | A A G D V N T E | EKSVLYS | NRAACYL | K D G N C S L C I K |
| tr G1KLT9 G1KLT9_Chameleon | - SADLK R | AGNEQF | RHGQY | SQAAALY | GRALAVLEA | AGDANAE | EKSVLYS | NRAACYL | KDGNLSLCIK |
| tr H3A8Q6 H3A8Q6_Latimeria | SASELKO | AGNESF | KNGQY | GQAASLY | TEAIRLLEE | EKGAKNTE | ELSILYS | NRAACYL | KDGNCNECIK |
| tr Q/20B8 Q/20B8_Danio | SWIDLKC | AGNECH | RAGQY | GEAVILY | SQAIQQLER | C S GQ K K I E | DEGILYS | NRAASYL | KDGNCNECIK |
| trivvomiteelvvomitee_Gar | DVAQUER | AGNUCF | | I GEAASLY | SKAIQLQER | | ELDILID | NKAASYL | NUGINCIECVK |
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| sp Q15785 TOM34_HUMAN | MD <mark>s</mark> lgpe | W <mark>R</mark> L K L P | SIPLV | V P V S AQ K R | W <mark>nslps</mark> en- | HK EMAK | S K S K E T T | ATKNRVP | SAGDVEKARV |
| sp Q15785 TOM34_HUMAN Sequence Ruler | MD <mark>SLGP</mark> E | WRLKLP | SIPLN | | WNSLPSEN | HK EMAK 170 | SKSKETT | ATKNRVP 180 | SAGDVEKARV 190 |
| sp Q15785 TOM34_HUMAN Sequence Ruler sp Q9CYG7 TOM34_MOUSE | MDSLGPE | W <mark>RLKL</mark> P 40 WRLKLP | SIPLV 15 PIPVV | <mark>∕ P V S AQ K R</mark> 50 √ P V S AQ K R | WNSLPSEN 160 WNSLPSDN | <mark>HK EMAK</mark> 170 HK ET AK | <u>SKSKETT</u> TKSKEAT | ATKNRVP 180 ATKSRVP | SAGDVEKARV 190 SAGDVERAKA |
| sp[Q15785]TOM34_HUMAN Sequence Ruler sp[Q9CYG7]TOM34_MOUSE tr[E18GD1]E18GD1_BOVIN | MDSLGPE MDSLGPE MDSFGPE | W <mark>R L K L P</mark> 40 WR L K L P WR L K L P | SIPLV PIPVV SIPLV | VPVSAQKR 50 VPVSAQKR VPVSSQKR | WNSLPSEN 160 WNSLPSDN WECLPLEN | HK EMAK 170 HK ET AK HK EP SK | SKSKETT TKSKEAT SKSKGTT | ATKNRVP 180 ATKSRVP STKSRVP | SAGDVEKARV 190 SAGDVERAKA SAGDVERAKA |
| sp[Q15785]TOM34_HUMAN Sequence Ruler sp[Q9CYG7]TOM34_MOUSE tr[E18GD1]E18GD1_BOVIN tr[F15DR5]F15DR5_PIG | MDSLGPE MDSLGPE MDSFGPE MDSFGS | WRLKLP WRLKLP WRLKLP | SIPLV PIPVV SIPLV SIPLV | V P V S AQ K R 50 V P V S AQ K R V P V S AQ K R V P V S AQ K R | WNSLPSEN 160 WNSLPSDN WECLPLEN WECLPSEN | | SKSKETT TKSKEAT SKSKGTT SKSKETT | AT KNR VP 180 AT K SR VP ST K SR VP T AK SR VP | SAGDVEKARV 190 SAGDVERAKA SAGDVERAKA SAGDVERARA SAGDVERARV |
| sp[Q15785]TOM34_HUMAN Sequence Ruler sp[Q9CYG7]TOM34_MOUSE tr[E18GD1]E18GD1_BOVIN tr[F15DR5]F15DR5_PIG tr]F1P4X4]F1P4X4_CHICK | MDSLGPE MDSLGPE MDSFGPE MDSFGSE LEKHGVN | WRLKLP 40 WRLKLP WRLKLP WRLKLP | SIPLV 15 PIPVV SIPLV SIPLV PIPTV | VPVSAQKR 50 VPVSAQKR VPVSSQKR VPVSAQKR VPVSAQKR VPVSAQKR | WNSLPSEN 160 WNSLPSDN WECLPLEN WECLPSEN WSVPSAGSF | HK EMAK 170 HK ET AK HK EP SK RK ET AK PT GGAP PG | SKSKETT TKSKEAT SKSKGTT SKSKETT NTPRGDP | AT KNR VP 180 AT K SR VP ST K SR VP T AK SR VP G E SP S AQ | SAGDVEKARV 190 SAGDVERAKA SAGDVERAKA SAGDVERARV TAAGIERAQT |
| sp[Q15785]TOM34_HUMAN Sequence Ruler sp[Q9CYG7]TOM34_MOUSE tr[E1BGD1]E1BGD1_BOVIN tr[F12DR5]F1SDR5_PIG tr[F1P4X4]F1P4X4_CHICK tr[V8P625]V8P625_Cobra | MDSLGPE MDSLGPE MDSFGPE MDSFGSE LEKHGVN LEKDGLQ | WRLKLP 40 WRLKLP WRLKLP WRLKLP WRLKLP WRLKLP | SIPLV PIPVV SIPLV SIPLV PIPTV PIPTV | V P V S AQ K R 50 V P V S AQ K R V P V S SQ K R V P V S AQ K R V P V S AQ K R V P V S AQ R R | WNSLPSEN 160 WNSLPSDN WECLPLEN WECLPSEN WSVPSAGSE WEPPTGSQ | | SKSKETT TKSKEAT SKSKGTT SKSKETT NTPRGDP AKASNYV | AT KNR VP 180 AT K SR VP ST K SR VP T A K SR VP G E SP S AQ P V T NQ VP | SAGDVEKARV 190 SAGDVERAKA SAGDVERAKA SAGDVERARV TAAGIERAQT NAASSEQANM |
| sp[Q15785]TOM34_HUMAN Sequence Ruler sp[Q9CYG7]TOM34_MOUSE tr[E18GD1]E18GD1_BOVIN tr[F15DR5]F1SDR5_PIG tr[F14X44]F14X4_CHICK tr[V8P625]V8P625_Cobra tr[G1KLT9]G1KLT9_Chameleon | MDSLGPE MDSLGPE MDSFGPE MDSFGSE LEKHGVN LEKDGLC LØKDGLC | WRLKLP 40 WRLKLP WRLKLP WRLKLP WREKLP WRQKLP WRQKLP | PIPU PIPU SIPLV SIPLV PIPTV PIPTV PIPTV | VPVSAQKR 50 VPVSAQKR VPVSQKR VPVSAQKR VPVSAQRR VPVSAQRR VPVSAQRR | WNSLPSEN 160 WNSLPSDN WECLPLEN WECLPSEN WECLPSEN WSVPSAGSF WEPPTGSQ WEPPTGSQ | 170 HK EMAK HK ET AK HK EP SK RK ET AK GGAP PG E DT PR | SKSKETT TKSKEAT SKSKGTT SKSKETT NTPRGDP AKASNYV - KVNAAA | ATKNRVP 180 ATKSRVP STKSRVP TAKSRVP GESPSAQ PVTNQVP PVTNQVP | SAGDVEKARV 190 SAGDVERAKA SAGDVERAKA SAGDVERARV TAAGIERAQT NAASEQANM TAASEQANM |
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