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 μM) was tested at various Hsp40 concentrations in malachite green assay. (*B*) Firefly luciferase was chemically denatured, mixed with Hsp70 (1 μM), Hsp40 (2 μM), ATP (1 mM), and varying Hop concentrations, and then recovered luminescence was measured.

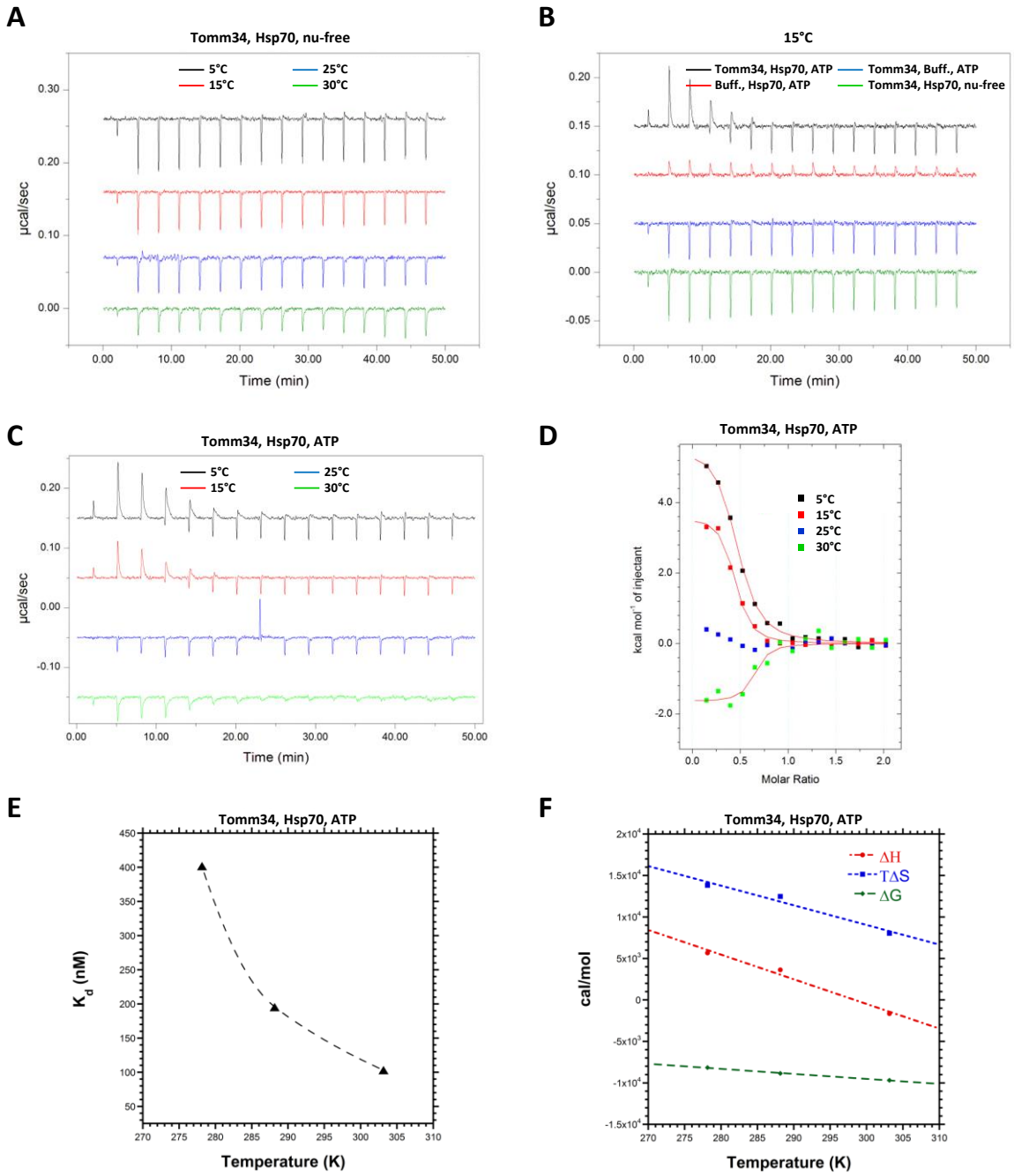
Supplemental Figure 3. The effect of I164D and D529A mutations, and interstitial deletion of 533-543 region on Hsp70 conformational activity and substrate binding. (*A*) Deuteration level differences of Hsp70 (I164D/D529A/Δ533-543) peptides in ATP-bound and nucleotide-free state after 1 h incubation 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 μM and 30 nM, respectively.

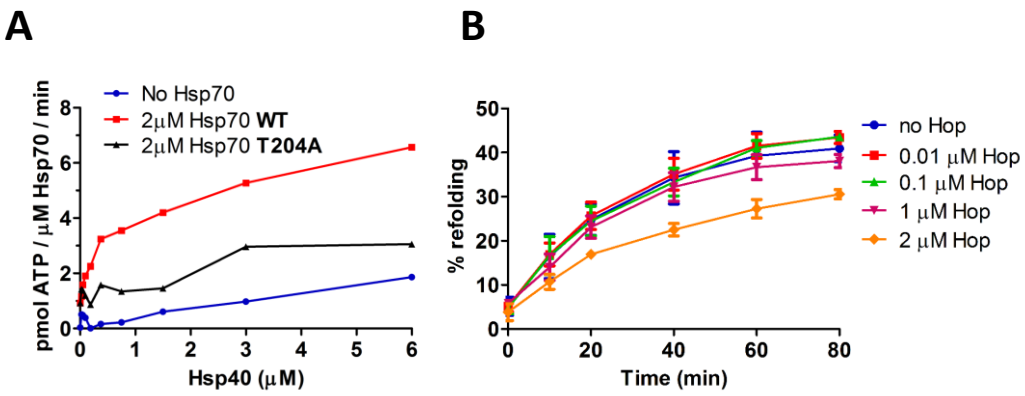
Supplemental Figure 4. The structure of Tomm34 is not affected by ATP. Deuteration level differences of Tomm34 peptides in the presence and absence of ATP (5 mM) after 10 min/3 h incubation in deuterated buffer.

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).

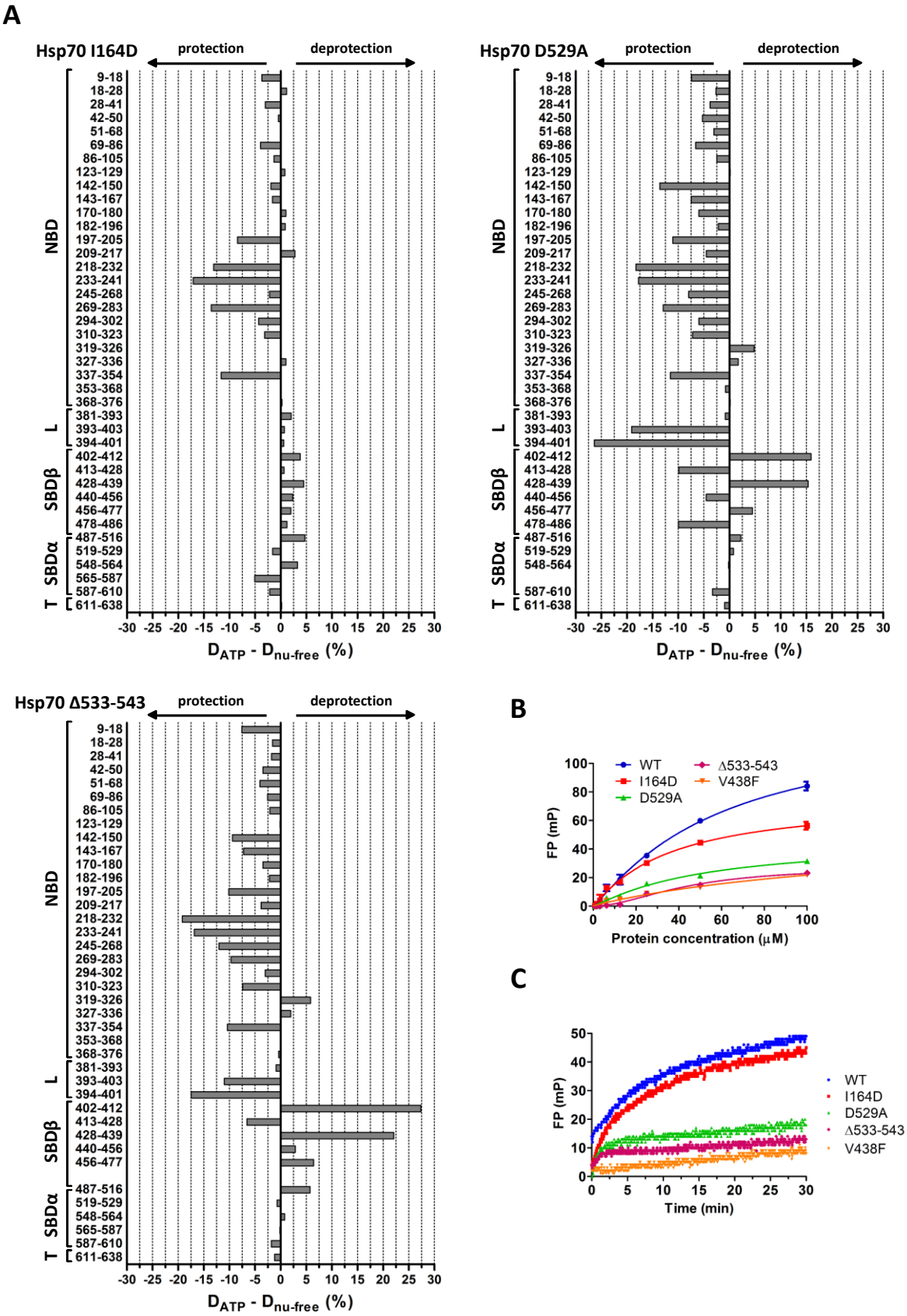
Supplemental Figure 1



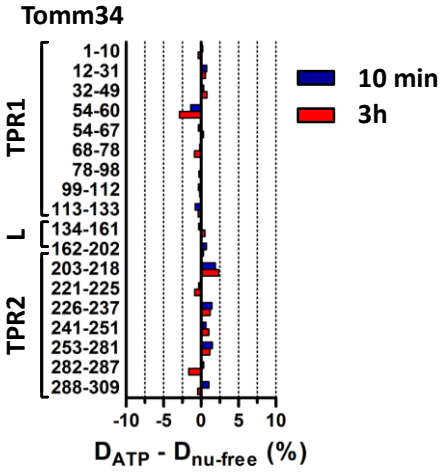
Supplemental Figure 2



Supplemental Figure 3

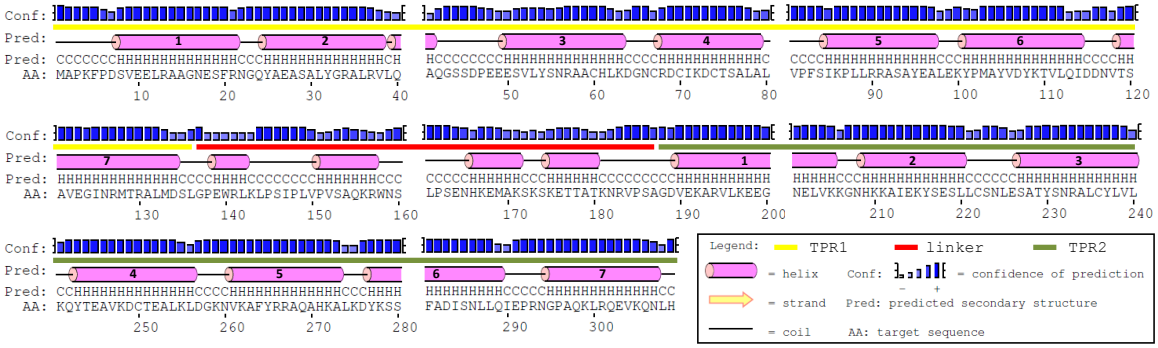


Supplemental Figure 4



Supplemental Figure 5

A



B