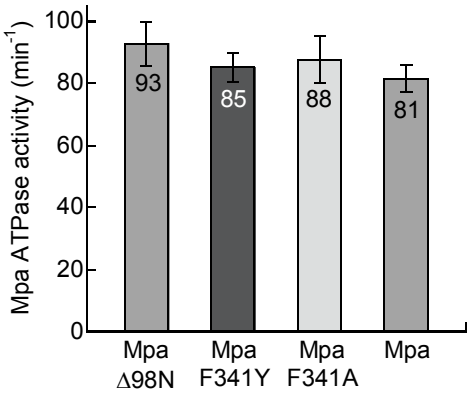
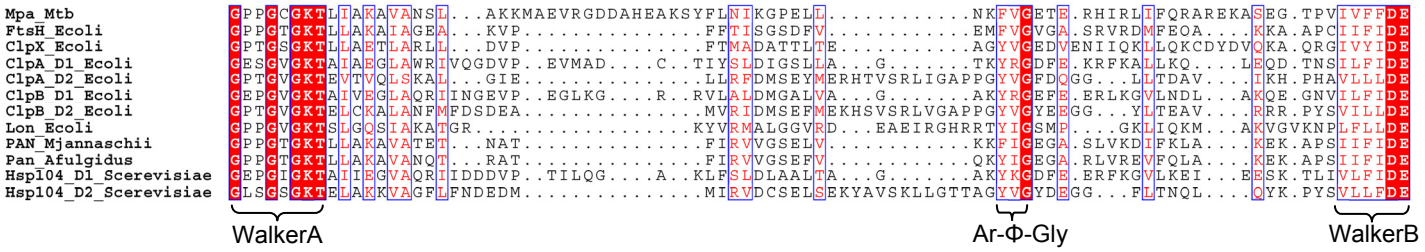
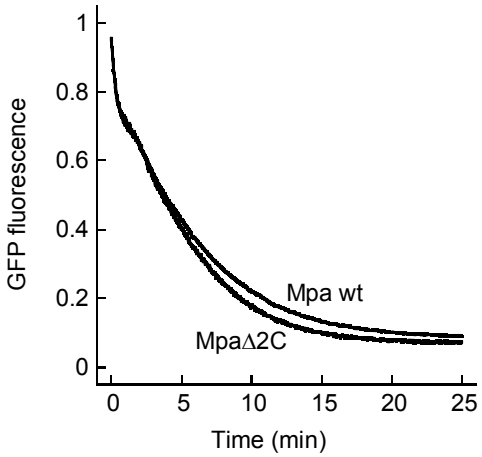


Supplementary Figure S1: ATPase activity of different Mpa-His_6 variants. Error bars represent s.d. from three experiments. The Mpa ATPase activity is calculated per hexamer. Note that the ATPase activity of wild-type Mpa is reduced to about 50% if the His_6-tag is present (compare to Figure 3D). Nevertheless, Mpa-His_6 is still active in unfolding Pup-GFP (Figure 2 “Mpa F341Y” and data not shown).

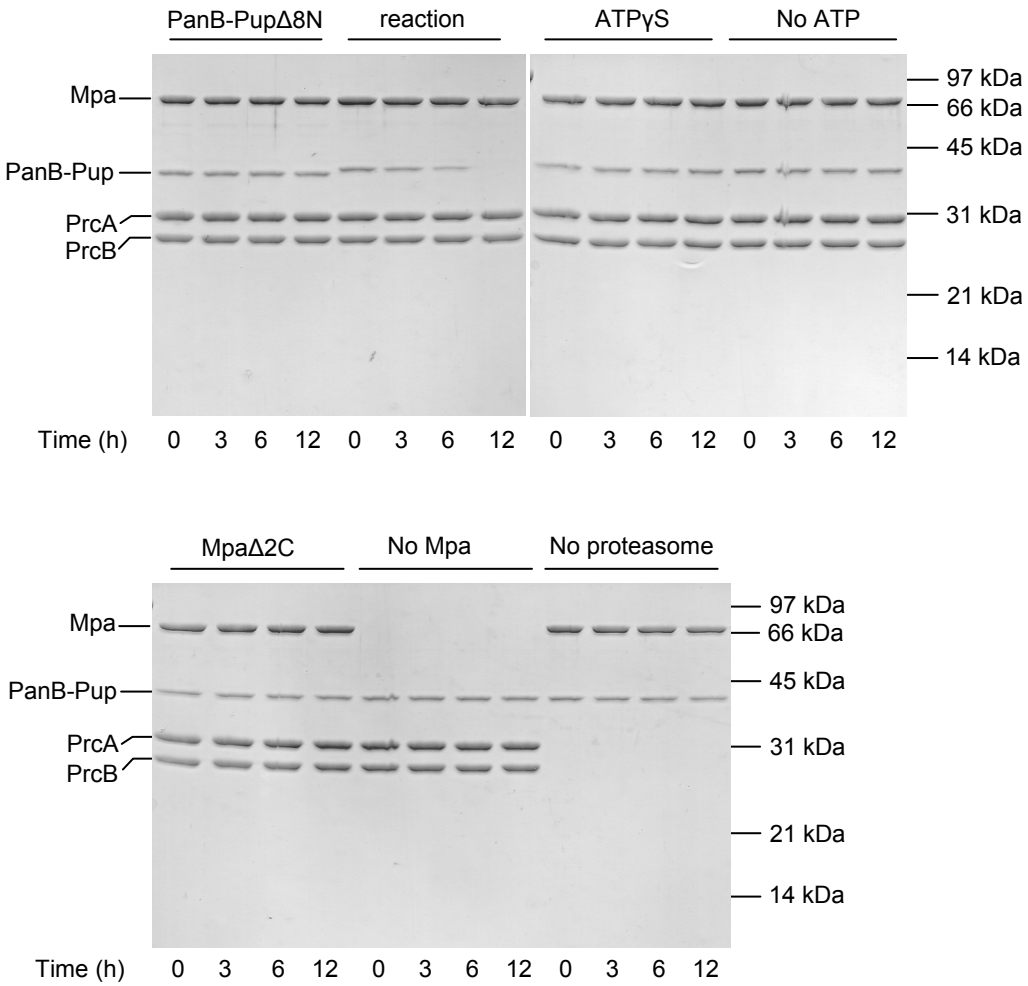


Supplementary Figure S2: Alignment of Mpa with different AAA-ATPases. The regions from the WalkerA to the WalkerB motif were aligned using TCoffee (Notredame *et al*, 2000). Sequences used are: *M. tuberculosis* Mpa (Mpa_Mtb), *E. coli* FtsH (FtsH_Ecoli), *E. coli* ClpX (ClpX_Ecoli), *E. coli* ClpA (ClpA_Ecoli), *E. coli* ClpB (ClpB_Ecoli), *E. coli* Lon (Lon_Ecoli), *M. jannaschii* PAN (PAN_Mjannaschii), *A. fulgidus* PAN (PAN_Afulgidus), *S. cerevisiae* Hsp104 (Hsp104_Scerevisiae). “D1“ and “D2“ refer to the first and second AAA-module if applicable, respectively.

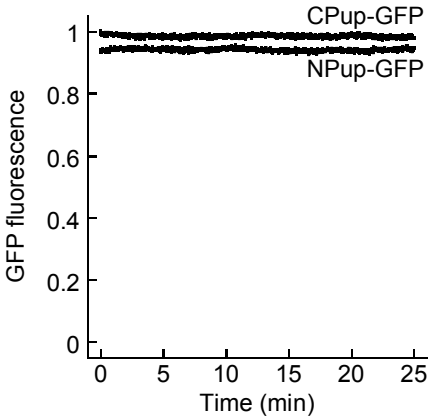
Notredame C, Higgins DG, Heringa J (2000) T-Coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302:** 205-217.



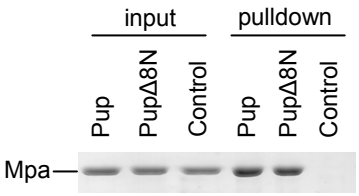
Supplementary Figure S3: MpaΔ2C is not impaired in its unfolding activity. MpaΔ2C and wild-type Mpa ("Mpa wt") were incubated with Pup-GFP in presence of GroELtrap and ATP.



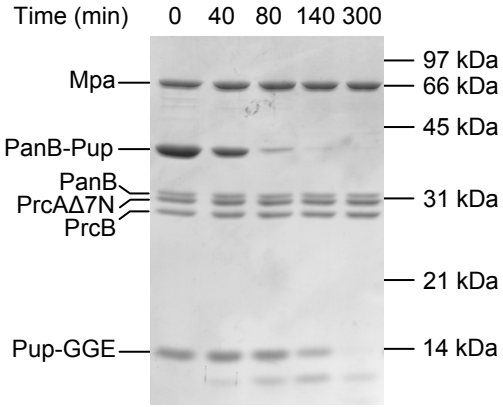
Supplementary Figure S4: Degradation of PanB-Pup by Mpa and the wild-type proteasome. PanB-Pup (1 μM) was incubated with Mpa (0.2 μM) and wild-type proteasome (0.2 μM) in presence of ATP (5 mM) and ATP regeneration system (termed “reaction”). The following control experiments were performed: PanB-PupΔ8N instead of PanB-Pup (“PanB-PupΔ8N”); MpaΔ2C instead of Mpa (“MpaΔ2C”); ATPγS (1 mM) instead of ATP (“ATPγS”); in absence of ATP (“No ATP”); in absence of Mpa (“No Mpa”); or in absence of proteasome (“No proteasome”). The reactions were carried out at 23 °C in 50 mM Hepes pH 7.5, 10 mM $MgCl_2$,1 mM DTT supplemented with 40 mM phosphocreatine and 0.4 U/mL creatine phosphokinase and analyzed by SDS-PAGE and Coomassie-staining. “PrcA” and “PrcB” refer to proteasomal alpha- and beta-subunits, respectively. The proteasome used in this experiment was reconstituted from inclusion bodies.



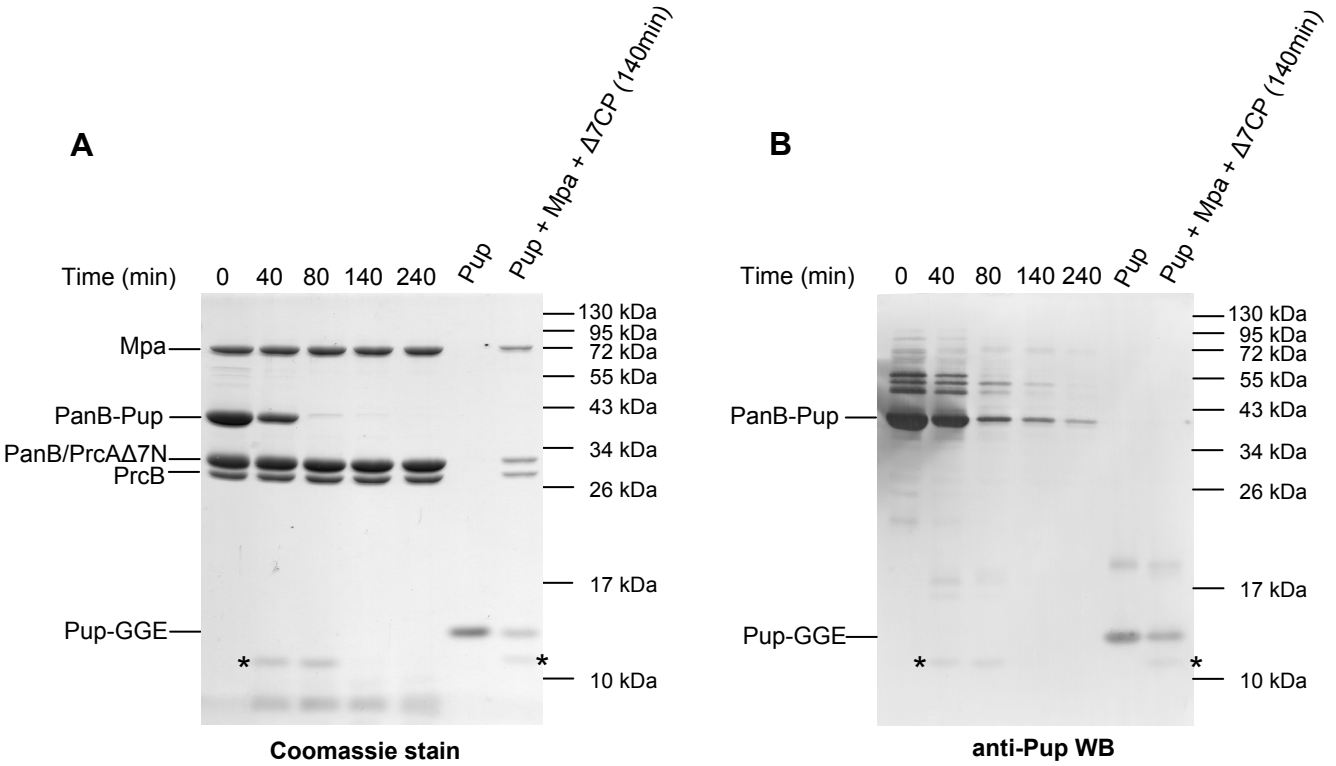
Supplementary Figure S5: Mpa does not unfold the fusion constructs in which either residues 1-34 of Pup are fused to GFP ("NPup-GFP") or residues 35-64 of Pup are fused to GFP ("CPup-GFP"). The reaction was performed in presence of GroELtrap and ATP.



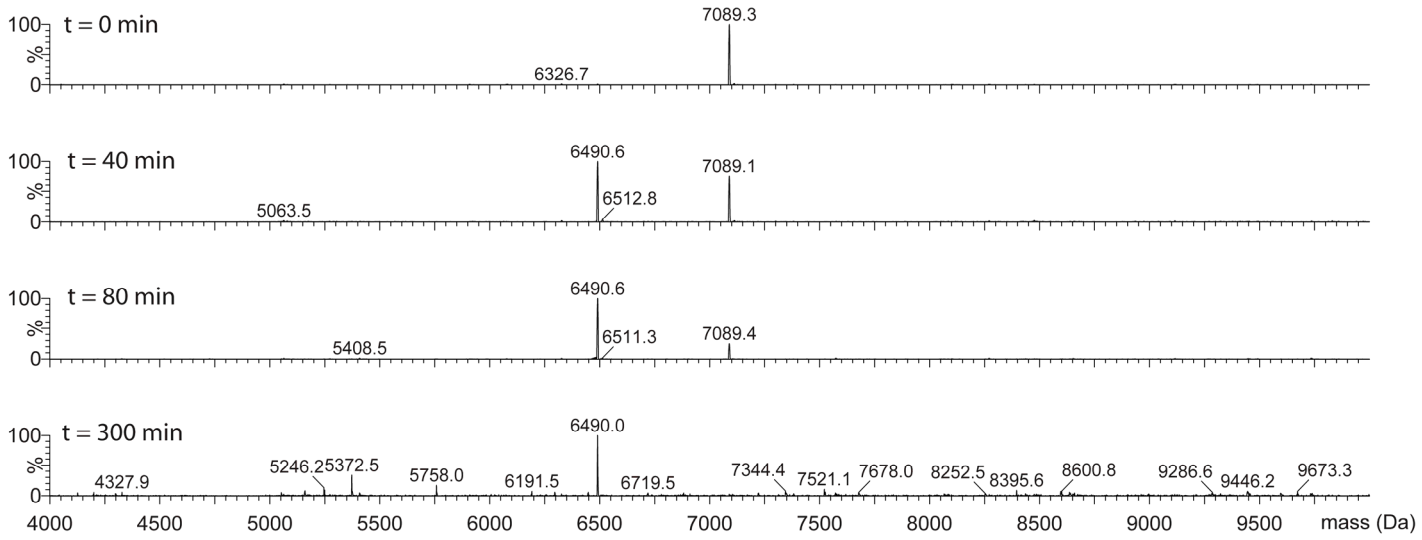
Supplementary Figure S6: PupΔ8N binds to Mpa. Pulldown with beads decorated with wild-type Pup (PupGGQ) or PupΔ8N or empty control beads on Mpa analyzed by SDS-PAGE and Coomassie-staining. 5% of the sample (“input”) was withdrawn before addition of beads.



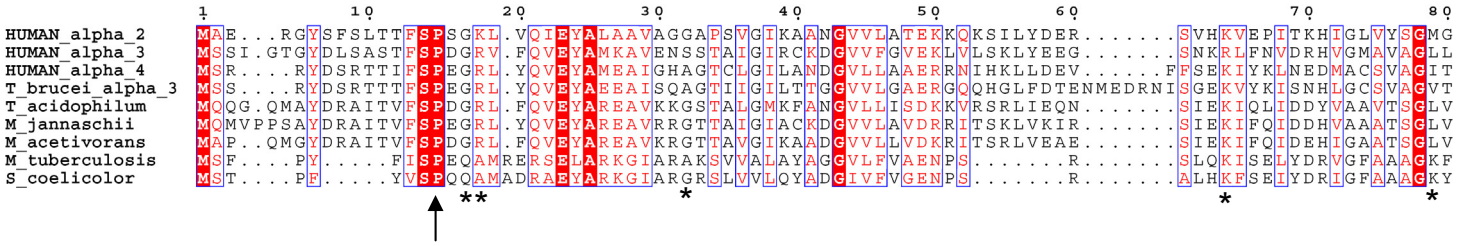
Supplementary Figure S7: Competition between degradation of a pupylated substrate and degradation of free Pup. Mpa (0.2 µM) and open-gate proteasome (0.1 µM) were incubated with PanB-Pup (10 µM) and free Pup-GGE (10 µM) in presence of ATP (5 mM) and ATP regeneration system. The reaction was analyzed by SDS-PAGE and Coomassie-staining. “PrcB” refers to the proteasomal beta-subunit, “PrcAΔ7N” to the proteasomal alpha subunit truncated by the 7 N-terminal residues.



Supplementary Figure S8: Pup is not recycled upon degradation of pupylated substrates. (**A**) Degradation of PanB-Pup by Mpa (0.3 μM) and open-gate proteasome (Δ7CP) (0.3 μM) in presence of ATP analyzed by SDS-PAGE and Coomassie-staining. For comparison, the amount of Pup (PupGGE) if Pup were recycled is loaded in lane 6. Lane 7 shows degradation of Pup (10 μM) by Mpa (0.1 μM) and Δ7CP (0.1 μM) in presence of ATP at t=140min (compare Figure 5). (**B**) SDS-PAGE gel described in (A) analyzed by anti-Pup Western-blot. The asterisks denote a transient degradation intermediate, representing a Pup-fragment truncated after Tyr58 (see Supplementary Figure S9). “PrcB” refers to the proteasomal beta-subunit, “PrcAΔ7N” to the proteasomal alpha subunit truncated by the 7 N-terminal residues.



Supplementary Figure S9: Degradation of Pup by Mpa and open-gate proteasome (Δ7CP) analyzed by ESI-MS. Pup-GGE (20 µM) was incubated with Mpa (0.2 µM) and Δ7CP (0.2 µM) in presence of ATP (5 mM) and ATP regeneration system. Aliquots were withdrawn at the indicated time points and the reaction was terminated by addition of EDTA and Pup was separated from Mpa and Δ7CP by ultrafiltration (50 000 MWCO). The theoretical mass of Pup-GGE is 7089.4 Da (containing an additional Gly-Ser stemming from the TEV-cleavage site). The 6490.6 Da fragment corresponds to Pup truncated after Tyr58 (theoretical mass 6490.7 Da). After 300 min the fragment is no longer visible in SDS-PAGE (compare Figure 5 and note the S/N-ratio at t = 300 min).



Supplementary Figure S10: Alignment of the N-terminal region of proteasomal alpha-subunits from various organisms using TCoffee. The sequences used are (top to bottom): human alpha subunit type 2, human alpha subunit type 3, human alpha subunit type 4, *Trypanosoma brucei* alpha subunit type 3 (all eukaryotes), *Thermoplasma acidophilum*, *Methanococcus jannaschii*, *Methanosarcina acetivorans* (all archaea), *Mycobacterium tuberculosis*, *Streptomyces coelicolor* (both bacteria). The arrow marks the conserved proline of the reverse turn loop that was shown to be displaced upon conversion from the closed to the open-gate conformation. Residues of the inter-subunit pocket of neighbouring alpha-subunits from *Thermoplasma acidophilum* involved in binding of the C-terminal peptide of PAN are marked with an asterisk (Rabl *et al*, 2008; Yu *et al*, 2009).

Supplementary Table I

Mpa constructs	
NdeI_mpa_fw	5′-GGATCCCATATGGGTGAGTCAGAGCGTTC-3′
BamHI_mpa_rev	5′-ATGCGGATCCTCACAGGTACTGGCCGAGGTTGG-3′
BamHI_mpaHis6_rev	5′-GCATGCGGATCCTCAATGATGATGATGATGATGCAGGTACTGGCCGAGGTTGG-3′
BspHI_mpaΔ98N_fw	5′-GATCGATCATGAGTGGCTACGGCGTCCTGCTGG-3′
mpa_F341A_fw	5′-CGAGCTGCTGAACAAAGCGGTCGGGGAAACGGAACGCCAC-3′
mpa_F341A_rev	5′-GTGGCGTTCCGTTTCCCCGACCGCTTTGTTCAGCAGCTCG-3′
mpa_F341Y_fw	5′-CGAGCTGCTGAACAAATACGTCGGGGAAACGGAACGCCAC-3′
mpa_F341Y_rev	5′-GTGGCGTTCCGTTTCCCCGACGTATTTGTTCAGCAGCTCG-3′
mpa_Δ2C_fw	5′-GTCCAACCTGGGCCAGTAGGGATCCGAATTCGAG-3′
mpa_Δ2C_rev	5′-CTCGAATTCGGATCCCTACTGGCCCAGGTTGGAC-3′
Pup-GFP constructs	
BamHI_pup_fw	5′-CTAGGGATCCATGGCGCAAGAGCAGACCAAGCG-3′
SacI_pup_rev	5′-GATCGAGCTCTCCGCCCTTTTGGACGTATGCGCG-3′
SacI_N-pup_rev	5′-GATCGAGCTCGGTCAACTTTTCGCGACGCTCCTGG-3′
BamHI_C-Pup_fw	5′-GATGGGATCCGAGACCGACGATCTGCTAGACGAAATC-3′
BamHI_pupΔ8N_fw	5′-GATAGGATCCGGCGGTGGAGGTGGCGATGATGAC-3′
BamHI_pupΔ19N_fw	5′-GATAGGATCCGGCAGCACTGCAGCGGGCCAG-3′
BamHI_NLep-pup_fw	5′-GATAGGATCCATGCTGAACGGCGAAGATGTCGTAGGCGGTGGAGGTGGCGATGATGAC-3′
BamHI_NCglu-pup_fw	5′-GATAGGATCCATGAATGCCAAACAGACCCAGATCGGCGGTGGAGGTGGCGATGATGAC-3′
BamHI_NSco-pup_fw	5′-GATAGGATCCATGGCAACCAAAGATACCGGCGGTGGAGGTGGCGATGATGAC-3′
BamHI_linker1-pup_fw	5′-GATAGGATCCTCTAACGGTGCTTCTGAATCTGGCGGTGGAGGTGGCGATGATGAC-3′
BamHI_linker2-pup_fw	5′-GATAGGATCCAGCGAAACCAGCAGTGCAAGTGGAGGCGGTGGAGGTGGCGATGATGAC-3′
SacI_gfp_fw	5′-GATCGAGCTCATGAGTAAAGGAGAAGAACTTTTCACTGGAG-3′
HindIII_gfp_rev	5′-GATCAAGCTTTTATTTGTAGAGTTCATCCATGCCATGTG-3′
Pup constructs	
BamHI_pupΔ8N_fw	5′-GATAGGATCCGGCGGTGGAGGTGGCGATGATGAC-3′
SacI_pupSTOP_rev	5′-GATCGAGCTCTTACTCTCCGCCCTTTTGGACGTATGCGCG-3′
PanB	
NcoI_panB_fw	5′-GATGCCATGGCTGAGCAGACTATCTATGGGGCC-3′
EcoRI_panBStrep_rev	5′-GACTGAATTCCTACTTTTCGAACTGAGGATGGCTCCAGAAACTGTGTTCGTCAGCGG-3′
Proteasome	
BspHI_prcA_fw	5′-GATCGATCATGAGTTTTCCGTATTTCATCTCGC-3′
BspHI_prcAΔ7N_fw	5′-GATCGATCATGAGTCCTGAGCAGGCGATGCGC-3′
EcoRI_prcA_rev	5′-ATCCATGAATTCTCAGCCCGACGATTCGCC-3′
NdeI_prcBΔpro_fw	5′-GGATCCCATATGACCACCATTGTCGCGCTGAAATAC-3′
EcoRV_prcBStrep_rev	5′-GGATCCGATATCTCACTTTTCGAACTGAGGATGTGACCACTTCTCACCGCCATCGGAGC-3′
HindIII_prcB_rev	5′-GATCAAGCTTTCACTTCTCACCGCCATCGGAGCC-3′

Supplementary Table I Primer sequences of constructs used in this study.