Characterization of the Binding Interaction Between the Oncoprotein Gankyrin and a Grafted S6 ATPase

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Table of Contents

Materials and Methods

Materials

All chemicals obtained from Sigma-Aldrich unless specified
LB Miller Broth, Fisher
5-alpha chemically competent *E. coli*, NEB
BL21 (DE3) chemically competent *E. coli*, NEB
Agar, Fisher
Carbenicillin, GoldBio Technology
Restriction Enzymes, NEB
Kanamycin, GoldBio Technology
Isopropyl-β-D-1-thiogalactopyranoside (IPTG), GoldBio Technology
Snakeskin Dialysis Tubing 10K MWCO, Thermo Scientific
Quick Ligation Kit, NEB
Q5 High Fidelity DNA Polymerase, NEB
Oligonucleotides, IDT
Miniprep Kit, OMEGA
PageRuler Prestained Protein Ladder, Thermo Scientific
12% Ready Gel precast gels, Biorad
Modified Lowry Protein Assay Kit, Pierce
cOmplete ULTRA Tablets, Roche

All water was obtained from a Milli-Q water purification system.

Instrumentation

Sonifer W-350 cell disruptor, Branson
J2-21 centrifuge, Beckman Coulter
MJ mini gradient thermal cycler, Biorad
iTC200, Microcal (Malvern)
Molecular imager gel doc XR+ system, Biorad
Innova 42/42R incubator shakers, New Brunswick Scientific
Circular dichroism spectrometer, Aviv model 202
NanoDrop 2000 UV-Vis Spectrophotometer, Thermo Scientific

Experimental Data

Protein Purification

Grafted FtsH-S6 ATPase was overlapped and amplified by PCR using oligonucleotides, and cloned into a pET plasmid using restriction enzymes BamHI and PacI, resulting in a N-terminally His_6 tagged construct, which was confirmed by DNA sequencing (all constructs in this manuscript were confirmed by GENEWIZ, South Plainfield, NJ). FtsH-S6 ATPase mutants were made using site-directed mutagenesis. These constructs were transformed into BL21s (DE3). Gankyrin was cloned into a pET plasmid using restriction enzymes NcoI and PacI, resulting in a C-terminally His_6 tagged construct, and transformed into BL21s (DE3). Gankyrin mutants were made using site-directed mutagenesis, and transformed into BL21s (DE3). Cells were grown in 1-2.5 L LB cultures containing carbenicillin at 37 °C to OD600= ~0.6 and induced with 1 mM IPTG at 25 °C for 8 hrs. Cells were then collected by centrifugation, resuspended in phosphate buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl) with protease inhibitor tablets and stored at -20 °C. Frozen pellets were thawed and sonicated for 2 minutes. The lysate was cleared by centrifugation (15000 rpm, 30 min.) and the supernatant was mixed with 1 mL of Ni-NTA agarose resin for 1 hour. The resin was collected by centrifugation (4950 rpm, 5 min.). The resin was washed with 50 mL of buffer containing 20 mM imidazole, followed by 10 mL with 50 mM imidazole. The protein was then eluted with 5 mL buffer containing 400 mM imidazole. The proteins were dialyzed against buffer and analyzed for purity by SDS-PAGE. Purified proteins were quantified using absorbance at 280nm and confirmed with a modified Lowry Assay.

Lysate Ni-NTA Pulldown Assay

Gankyrin variants were cloned into MCS1 of pET using restriction enzymes BamHI and HindIII, resulting in N-terminal His_6-tagged constructs. FtsH-S6 ATPase constructs were cloned into MCS2 of pET using the restriction enzymes NdeI and PacI. Completed constructs were transformed into BL21s (DE3). Cells containing the co-expressed pair were inoculated and induced as described previously. Cells were spun down and resuspended in lysis buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 1 mM DTT) lysed by sonication, and spun down to remove cell debris. Cleared lysate was incubated with 100 µL Ni-NTA agarose resin for 1 hour. Ni-NTA agarose was washed with 5mL lysis buffer and 5mL lysis buffer with 20 mM imidazole. Proteins were eluted with lysis buffer containing 400 mM imidazole. The pulldown was analyzed by SDS-PAGE.

Isothermal Titration Calorimetry

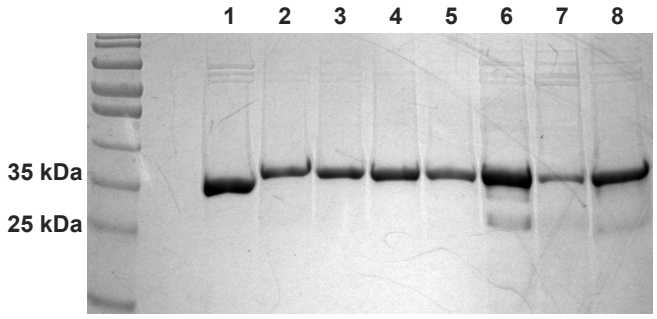
Isothermal titration calorimetry was performed using a MicroCal iTC200 calorimeter maintained at 25 °C. All proteins were purified as described previously and dialyzed

extensively in phosphate buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 2.5 mM 2-mercaptoethanol). FtsH-S6 ATPase variants were placed in the sample cell at concentrations ranging from 15-18 μM (30 μM for FtsH-S6 R338A/R339A/R342A), and 150-180 μM (500 μM for FtsH-S6 R338A/R339A/R342A interaction) of Gankyrin variants were titrated in 2.49 μL increments (16 injections total), with an initial injection of 0.2 μL, at 180 sec intervals using a stirring speed of 750 rpm. Heats of dilution were measured in the same manner described above, separately titrating buffer into buffer and Gankyrin (and all mutants) into buffer. Data were analyzed using Origin7.0 (MicroCal, iTC200) using a one set of sites binding model for fitting. All data were reference subtracted by subtracting the mean heat of dilution from each data point.

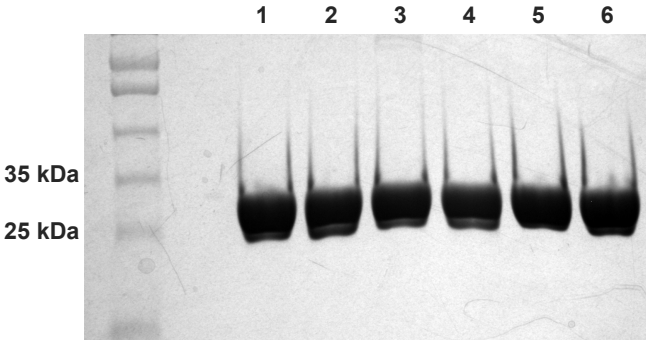
Circular Dichroism

Proteins were purified as described above. Separately, each protein was diluted to 5 μM in phosphate buffer (20 mM sodium phosphate pH 7.4, 150 mM NaCl, 2.5 mM 2-mercaptoethanol) and placed in a quartz cuvette with a pathlength of 0.2 cm. Data were collected on an Aviv model 202 circular dichroism spectrometer. Wavelength data were taken from scans of 250 nm to 200 nm in 1 nm steps at 25 °C.

Figure S1. SDS-PAGE of Purified Proteins. PAGE analysis of purified grafted FtsH-S6 ATPase and Gankyrin, including mutants thereof, used for ITC analysis.



Lane 1: wt FtsH
Lane 2: FtsH-S6
Lane 3: FtsH-S6 R342A
Lane 4: FtsH-S6 R338A/R342A
Lane 5: FtsH-S6 R338A/R339A/R342A
Lane 6: FtsH-S6 E356A/E357A
Lane 7: FtsH-S6 D359A/D362A
Lane 8: FtsH-S6 K397E



Lane 1: Gankyrin
Lane 2: Gankyrin R41A
Lane 3: Gankyrin K116A
Lane 4: Gankyrin R41A/K116A
Lane 5: Gankyrin D39A/D71A
Lane 6: Gankyrin E182A

Figure S2. ITC of wt-FtsH and Gankyrin. ITC evaluation of wild-type FtsH from *E. coli* and Gankyrin. No appreciable binding was observed.

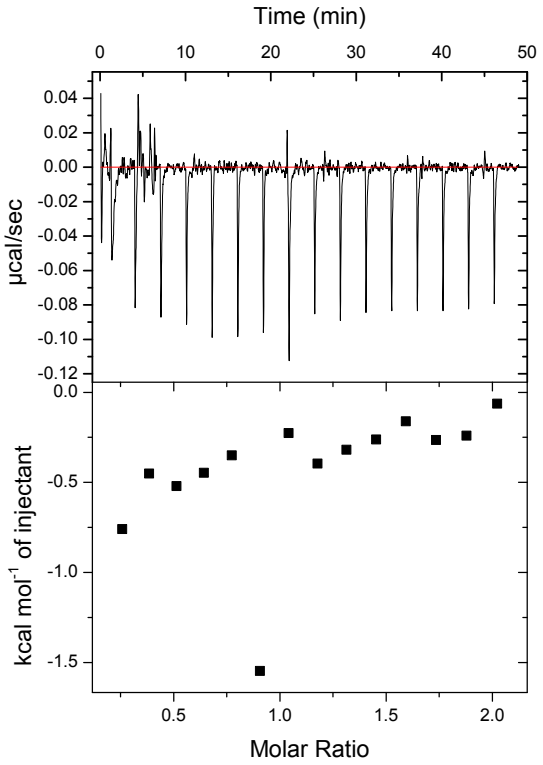


Figure S3. ITC Data (summarized in Table 1). Representative ITC binding isotherms involving Gankyrin and grafted FtsH-S6 ATPase, and specific mutants thereof.

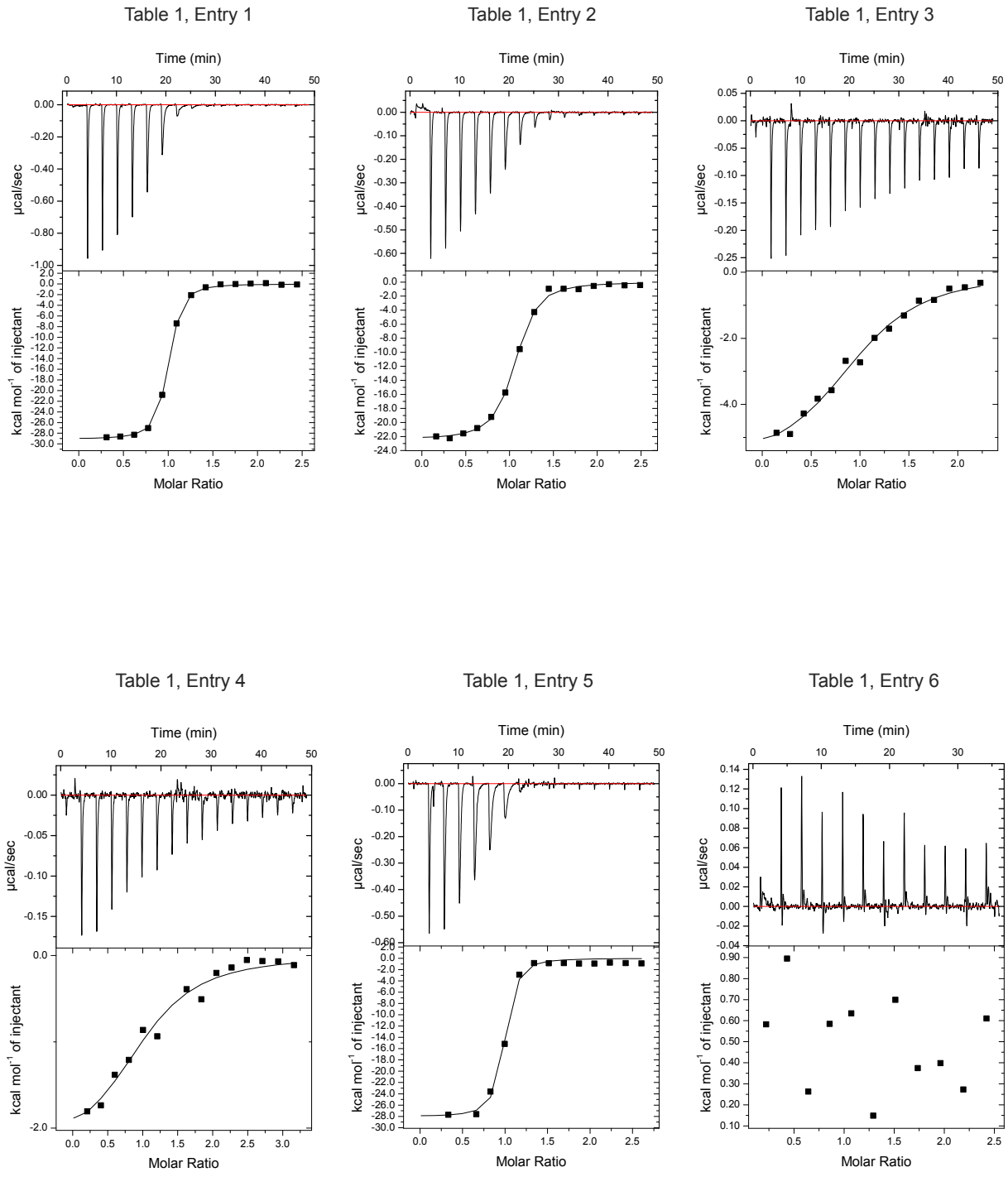


Table 1, Entry 7

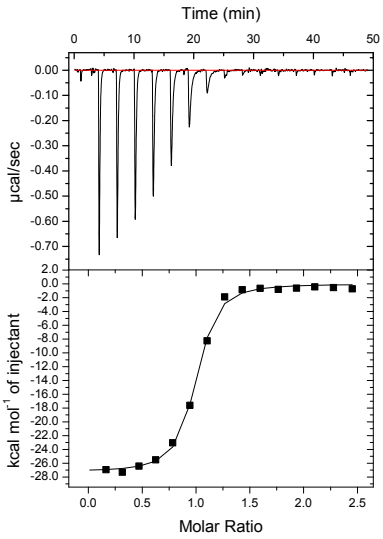


Table 1, Entry 8

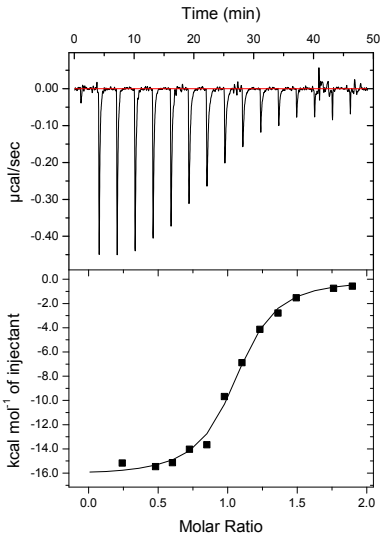


Table 1, Entry 9

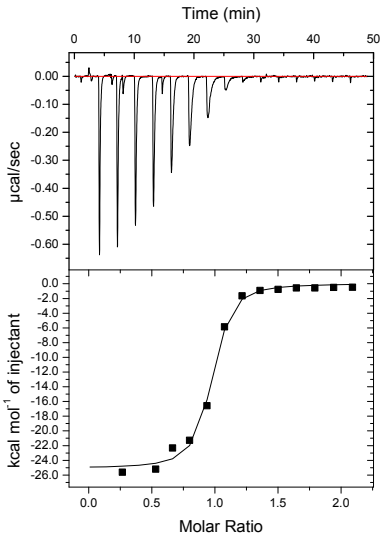


Table 1, Entry 10

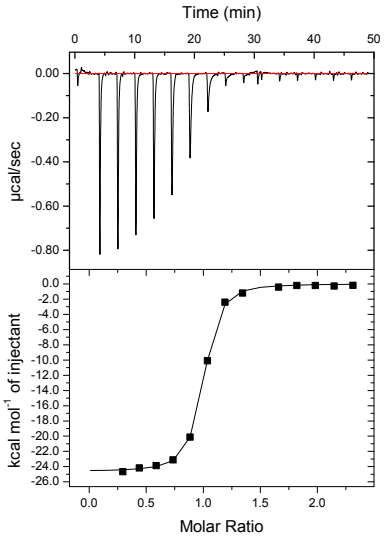


Table 1, Entry 11

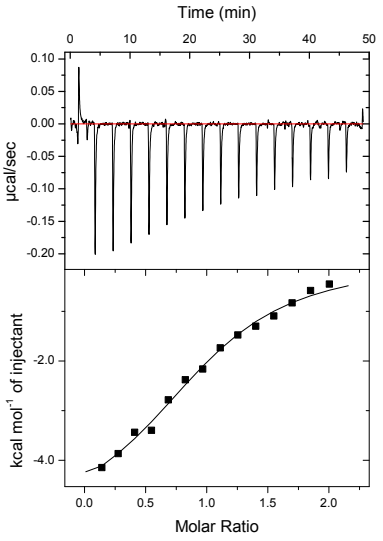


Table 1, Entry 12

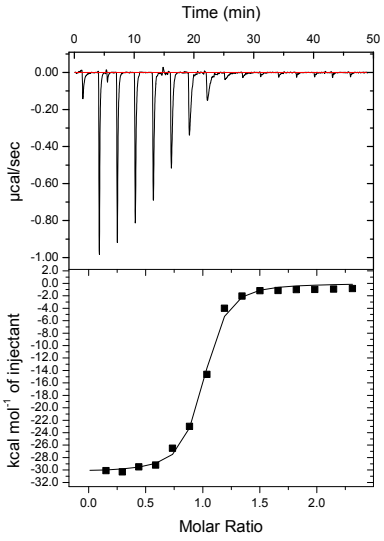


Figure S4. ITC Binding Stoichiometry. N-values for each entry in Table 1. All error represents the standard deviation of three separate experiments.

Entry	gankyrin mutant	FtsH-S6 ATPase mutant	N-value
1	wt-gankyrin	wt-FtsH-S6 ATPase	0.93±0.01
2	wt-gankyrin	R342A	1.02±0.02
3	wt-gankyrin	R338A/R342A	0.99±0.03
4	wt-gankyrin	R338A/R339A/R342A	1.01±0.04
5	wt-gankyrin	E356A/E357A	0.93±0.02
6	wt-gankyrin	D359A/D362A	no binding
7	wt-gankyrin	K397E	0.94±0.01
8	R41A	wt-FtsH-S6 ATPase	0.99±0.04
9	K116A	wt-FtsH-S6 ATPase	0.91±0.01
10	D39A/D71	wt-FtsH-S6 ATPase	0.93±0.02
11	R41A/K116A	wt-FtsH-S6 ATPase	0.97±0.05
12	E182A	wt-FtsH-S6 ATPase	0.97±0.02

Figure S5. Protein Sequences.

Gankyrin

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQDSRTALHWACSAGHTEIVEFL
LQLGVPVNDKDDAGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAASK
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT
PLHLACDEERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

Gankyrin R41A

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQDS**A**TALHWACSAGHTEIVEFL
LQLGVPVNDKDDAGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAASK
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT
PLHLACDEERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

Gankyrin K116A

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQDSRTALHWACSAGHTEIVEFL
LQLGVPVNDKDDAGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAAS**A**
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT
PLHLACDEERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

Gankyrin R41A/K116A

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQDS**A**TALHWACSAGHTEIVEFL
LQLGVPVNDKDDAGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAAS**A**
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT
PLHLACDEERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

Gankyrin D39A/D71A

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQ**A**SRTALHWACSAGHTEIVEFL
LQLGVPVNDKD**A**AGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAASK
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT
PLHLACDEERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

Gankyrin E182A

MEGCVSNIMICNLAYSGKLDELKERILADKSLATRTDQDSRTALHWACSAGHTEIVEFL
LQLGVPVNDKDDAGWSPLHIAASAGRDEIVKALLVKGAHVNAVNQNGCTPLHYAASK
NRHEIAVMLLEGGANPDAKDHYDATAMHRAAAKGNLKMVHILLFYKASTNIQDTEGNT

PLHLACD**A**ERVEEAKFLVTQGASIYIENKEEKTPLQVAKGGLGLILKRLAEGEEASMGH
HHHHH*

wt FtsH

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPDVRGREQILKVHMRRVPLAPDIDAAIIARGTPGFSGADL
ANLVNEAALFAARGNKRVVSMVEFEKAKDKIMMGA*

FtsH-S6

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPDRRGKRQIFSTHTSKMNLSEEVDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 R342A

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPDRRGK**A**QIFSTHTSKMNLSEEVDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 R338A/R342A

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPD**A**RGK**A**QIFSTHTSKMNLSEEVDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 R338A/R339A/R342A

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPD**AA**GK**A**QIFSTHTSKMNLSEEVDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 E356A/E357A

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPDRRGKRQIFSTHTSKMNLS**AA**VDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 D359A/D362A

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
APCIIFIDEIDAVGRQRGAGLGGGHDEREQTLNQMLVEMDGFEGNEGIIVIAATNRPDV
LDPALLRPGRFDRQVVVGLPDRRGKRQIFSTHTSKMNLSEEV**A**LE**A**YVARPDKISGAD
INSICQESGMLAVRENRYIVLAKDFEKAYKTVIKKDEQEHEFYK*

FtsH-S6 K397E

MGSSHHHHHHSQDPLTEDQIKTTFADVAGCDEAKEEVAELVEYLREPSRFQKLGGKIP
KGVLMVGPPGTGKTLLAKAIAGEAKVPFFTISGSDFVEMFVGVGASRVRDMFEQAKKA
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LDPALLRPGRFDRQVVVGLPDRRGKRQIFSTHTSKMNLSEEVDLEDYVARPDKISGAD
INSICQESGMLAVRENRYIVL**A**EDFEKAYKTVIKKDEQEHEFYK*

Figure S6. Oligonucleotides used to prepare constructs for ITC. All DNA primers are listed 5' to 3'

FtsH-S6 ATPase overlap primers

FP1: CATGCCATGGGCAGCAGCCATCACCATCATCACCACAGCC

RP1: GTCTTCGGTCAGCGGATCCTGGCTGTGGTGATGATGGTGA

FP2: AGGATCCGCTGACCGAAGACCAGATCAAAACCACNTTCGC

RP2: CGTCGCAACCAGCAACGTCAGCGAANGTGGTTTTGATCTG

FP3: TGACGTTGCTGGTTGCGACGAAGCTAAAGAAGAAGTTGCT

RP3: CGCAGGTATTCAACCAGTTCAGCAACTTCTTCTTTAGCTT

FP4: GAACTGGTTGAATACCTGCGTGAACCGTCTCGTTTCCAGA

RP4: CGGGATTTTACCACCCAGTTTCTGGAAACGAGACGGTTCA

FP5: AACTGGGTGGTAAAATCCCGAAAGGTGTTCTGATGGTTGG

RP5: TTTTACCGGTACCCGGCGGACCAACCATCAGAACACCTTT

FP6: TCCGCCGGGTACCGGTAAAACCCTGCTGGCTAAAGCTATC

RP6: GGAACTTTAGCTTCACCAGCGATAGCTTTAGCCAGCAGGG

FP7: GCTGGTGAAGCTAAAGTTCCGTTCTTCACCATCTCTGGTT

RP7: GAACATTTCAACGAAGTCAGAACCAGAGATGGTGAAGAAC

FP8: CTGACTTCGTTGAAATGTTCGTTGGTGTTGGTGCTTCTCG

RP8: GTTCGAACATGTCACGAACACGAGAAGCACCAACACCAAC

FP9: TGTTCGTGACATGTTCGAACAGGCTAAAAAAGCTGCTCCG

RP9: TCGTCGATGAAGATGATGCACGGAGCAGCTTTTTTAGCCT

FP10: TGCATCATCTTCATCGACGAAATCGACGCTGTTGGTCGTC

RP10: ACCCAGACCAGCACCACGCTGACGACCAACAGCGTCGATT

FP11: AGCGTGGTGCTGGTCTGGGTGGTGGTCACGACGAACGTGA

RP11: GCATCTGGTTCAGGGTCTGTTCACGTTCGTCGTGACCACC

FP12: ACAGACCCTGAACCAGATGCTGGTTGAAATGGACGGTTTC

RP12: ATGATACCTTCGTTACCTTCGAAACCGTCCATTTCAACCA

FP13: GAAGGTAACGAAGGTATCATCGTTATCGCTGCTACCAACC

RP13: CGGGTCGAGAACGTCCGGACGGTTGGTAGCAGCGATAACG

FP14: GTCCGGACGTTCTCGACCCGGCTCTGCTGCGTCCGGGTCG

RP14: CAACAACCTGACGGTCGAAACGACCCGGACGCAGCAGAGC

FP15: TTTCGACCGTCAGGTTGTTGTTGGTCTGCCGGACCGCCGC

RP15: GAGAAAATCTGTCTCTTCCCGCGGCGGTCCGGCAGACCAA

FP16: GGGAAGAGACAGATTTTCTCCACTCACACTAGCAAGATGA

RP16: GTCAACCTCCTCAGAGAGGTTCATCTTGCTAGTGTGAGTG

FP17: ACCTCTCTGAGGAGGTTGACTTGGAAGACTATGTGGCCCG

RP17: CTCCTGAAATCTTATCTGGCCGGGCCACATAGTCTTCCAA

FP18: GCCAGATAAGATTTCAGGAGCTGATATTAACTCCATCTGT

RP18: GCCAACATTCCACTCTCCTGACAGATGGAGTTAATATCAG

FP19: CAGGAGAGTGGAATGTTGGCTGTCCGTGAAAACCGCTACA

RP19: GAAGTCCTTGGCCAGGACAATGTAGCGGTTTTCACGGACA

FP20: TTGTCCTGGCCAAGGACTTCGAGAAAGCATACAAGACTGT

RP20: CCTGCTCGTCCTTCTTGATGACAGTCTTGTATGCTTTCTC

FP21: CATCAAGAAGGACGAGCAGGAGCATGAGTTTTACAAGTGA

RP21: CCTTAATTAATCACTTGTAAAACTCATGCT

FtsH-S6 ATPase mutant primers

R342A FP: CCGGACCGCCGCGGGAAGGCACAGATTTTCTCCACTCAC

R342A RP: GTGAGTGGAGAAAATCTGTGCCTTCCCGCGGCGGTCCGG

R338A/R342A FP: GTTGTTGGTCTGCCGGACGCCCGCGGGAAGGCACAGATTTTCTCCACTCAC

R338A/R342A RP: GTGAGTGGAGAAAATCTGTGCCTTCCCGCGGGCGTCCGGCAGACCAACAAC

R338A/R339A/R342A FP:
GTTGTTGGTCTGCCGGACGCCGCCGGGAAGGCACAGATTTTCTCCACTCAC

R338A/R339A/R342A RP:
GTGAGTGGAGAAAATCTGTGCCTTCCCGGCGGCGTCCGGCAGACCAACAAC

E356A/E357A FP: GCAAGATGAACCTCTCTGCGGCGGTTGACTTGGAAGACTATG

E356A/E357A RP: CATAGTCTTCCAAGTCAACCGCCGCAGAGAGGTTCATCTTGC

D359A/D362A FP: CCTCTCTGAGGAGGTTGCCTTGGAAGCCTATGTGGCCCGGCCAG

D359A/D362A RP: CTGGCCGGGCCACATAGGCTTCCAAGGCAACCTCCTCAGAGAGG

K397E FP: CTACATTGTCCTGGCCGAGGACTTCGAGAAAGC

K397E RP: GCTTTCTCGAAGTCCTCGGCCAGGACAATGTAG

Gankyrin mutant primers

R41A FP: CTAGAACTGATCAGGACAGCGCAACAGCTTTGCACTGGGCATG

R41A RP: CATGCCCAGTGCAAAGCTGTTGCGCTGTCCTGATCAGTTCTAG

K116A FP: CACTCCATTATGCAGCTTCGGCGAATAGGCATGAGATTGCTG

K116A RP: CAGCAATCTCATGCCTATTCGCCGAAGCTGCATAATGGAGTG

D39A FP: GCTACTAGAACTGATCAGGCCAGCAGAACAGCTTTGCAC

D39A RP: GTGCAAAGCTGTTCTGCTGGCCTGATCAGTTCTAGTAGC

D71A FP: GCCAGTGAATGATAAAGATGCCGCAGGTTGGTCTCCTCTTC

D71A RP: GAAGAGGAGACCAACCTGCGGCATCTTTATCATTCACTGGC

E182A FP: CACTTAGCCTGTGATGCAGAGAGAGTGGAAGAG

E182A RP: CTCTTCCACTCTCTCTGCATCACAGGCTAAGTG

Gankyrin primers

Gankyrin FP (w/ NcoI cut site): CATGCCATGGAGGGGTGTGTGTCTAACATAATGATCTGTAACC

Gankyrin RP (w/ PacI cut site):
CCTTAATTAATTAGTGATGGTGGTGGTGATGACCCATAGAAGCCTCTTCACCTTCTGCTA