**Supporting Online Material**

## Materials and Methods

### Reagents

Synthetic signal peptides were ordered from Elim Biosciences. All peptides were purified to >90% purity and the molecular mass of the peptides was confirmed by mass spectrometry. In all cases, a single peak was present at the predicted mass. Soluble peptides used in this study are: FAM-F17 ((5-FAM)-IYC NNA QLA PYQ RTG RNV YD); ΔEspP-FAM (MKK HKR ILA LCF LGL LQS SYS FA K(5-FAM)-NH2); ΔEspP-AAKKKK (MKK HKR ILA LCF LGL LQS SYS AAK KKK). Peptide arrays were purchased from the MIT Biopolymers Laboratory. Purified Kar2 was a generous gift from the Brodsky lab.

### Purification of Ire1

Ire1 cLD (aa N111-E461) and the W426A and M229A, F285A, Y301A mutants were purified as GST fusion proteins similar to a previously described protocol (*1*). Briefly, plasmids encoding the GST-cLD were transformed into *E. coli* BL21-DE3* and grown in LB-ampicillin at 30ºC until $OD_{600}$= 0.7. Expression of GST-cLD was then induced with 0.3 mM IPTG at 18ºC for 16 hours. Cells were harvested at 6000xg for 10 minutes, and resuspended in Lysis Buffer (50 mM Tris pH 7, 10% glycerol, 250 mM NaCl, 4 mM DTT). Cells were lysed with 2 passages through the Avestin EmulsiFlex-C3 at 15,000 psi, and the supernatant was collected after 30 minutes at 30,000xg. The supernatant was batch bound to pre-washed glutathione sepharose for 2 hours, then poured into a column and washed with 200 mL Lysis Buffer. At this point, GST-cLD was either eluted with 20 mM glutathione, or the GST tag was removed by overnight incubation with Prescission Protease. In both cases the eluted proteins were applied to a MonoQ 10/10 ion exchange column equilibrated in Lysis Buffer and eluted with a linear gradient of Lysis Buffer with 1 M NaCl. Sample were then concentrated and further purified on a Superdex200 10/300 gel filtration column equilibrated in the Minimal Reaction Buffer (20 mM Tris pH 7, 250 mM NaCl, 2 mM $CaCl_2$, 2 mM TCEP). 10% glycerol was added for storage at -80 ºC. The concentration of protein was determined using the predicted extinction coefficient at 280 nm.

### Induction of the UPR

Induction of the UPR response was detected by flow cytometry analysis of the median FITC intensity of cells with an integrated pRS304 4xUPRE-GFP reporter or an integrated pRS303 *HAC1* mRNA splicing reporter as described previously (*2*). ER stress was induced by either treatment with 5 mM DTT, or galactose inducible expression of HA tagged CPY/CPY* or CPY* fragments fused to FKBP as a scaffold (CPY signal peptide-CPY fragment-FKBP-HDEL). To detect growth phenotypes under UPR inducing conditions, 5-fold dilutions of Cry1 Δ*ire1*::KanMX6 with either an empty CEN-ARS plasmid or with either $P_{IRE1}$-*IRE1* or $P_{IRE1}$-*ire1*ΔMFY were spotted on plates with and without 0.25 ug/mL tunicamycin.

### Immunoprecipitation of Ire1

Ire1 tagged at its C-terminus with 3xFLAG 6xHis was integrated into the genomic locus of BY4741 Δ*bar1*::KAN. HA-tagged CPY, CPY*, or CPY* fragments fused to FKBP as a scaffold (same as above) were expressed from $P_{GAL1}$ on a CEN-ARS plasmid. Yeast strains were grown in the appropriate synthetic media with 2% raffinose before a two-hour induction with 2% galactose. After the two-hour induction ($OD_{600}$ ~0.4), cells were collected at 10,000xg for 10 min, resuspended in IP buffer (20 mM HEPES, 250 mM NaCl, 2 mM $MgCl_2$, 1% Triton X-100), and protease inhibitors and frozen at -80º C. The frozen cells were lysed in a Retsch Mixer Mill for 5x3 min intervals at 15 Hz. After pelleting cell debris at 4000xg for 15 min, the supernatants were diluted to 6 µg/µl protein, and added to Invitrogen Dynabeads M270 conjugated to Sigma α-FLAG M2 antibodies. Dynabeads and supernatant were incubated for 4 hours at 4ºC, then washed 4x2 min with IP buffer with 0.05% Tween-20. Proteins were eluted in 40 µL of 250 µg/mL Sigma 3xFLAG peptide and observed by Western Blot with Sigma α-FLAG M2, Covance α-HA.11, and Affinity Bioreagents α-FKBP12.

**Peptide arrays**

Peptide arrays were purchased from the MIT Biopolymers Laboratory. The CPY* tiling arrays were composed of 18 mer peptides (GST-cLD$^{W426A}$) or 20 mer peptides (WT GST-cLD) that tiled through the CPY* sequence by 3 amino acids at a time. In the mutational arrays, peptides were synthesized to systematically mutate each amino acid in the core region of the F17 peptide or by alanine/glutamic acid scanning in the ΔEspP peptide. For the peptide array of chaperone substrates, peptides were taken from the literature (*3-6*), and shorter peptides were placed in tandem to keep the lengths of peptides similar.

The arrays were incubated in methanol for 10 minutes, then in Binding Buffer (50 mM Tris pH 7, 250 mM NaCl, 10% glycerol, 2 mM DTT) for 3x10 min washes. The arrays were then incubated for 1 hour at room temperature with 500 nM GST-cLD WT or W426A. The arrays were washed again for 3x10 min washes in Binding Buffer to remove any unbound GST-cLD. Using a semi-dry transfer apparatus, bound GST-cLD was transferred to a PVDF membrane and detected with Abcam α GST S tag 05 antibody. Arrays were stripped before re-use (*7*). The peptide array with Kar2 followed the same protocol, but used 50 mM Tris pH 7.4, 50 mM KCl, 10 mM $MgCl_2$, 2 mM TCEP as the binding buffer and rabbit α-Kar2 antibodies (Caroline Shamu).

In order to calculate the contribution of each amino acid to cLD binding, the intensity of each spot containing that amino acid were averaged. For example, Ala18 was present in 6 spots, so the intensity of those 6 spots were averaged and plotted for Ala18. The intensities were quantitated using ImageQuant Array analysis software with background subtraction for a spot containing no peptide. Average spot intensities were normalized to the maximum spot intensity for each array (*8*).

For the mutational arrays, the spot intensity of each mutated peptide was compared to the intensity of the wild-type peptide in that row or column which was set at 100%. A percent of WT binding above 100% in Figure 2C indicates that the mutation of the WT amino acid on the x-axis to the substituted amino acid indicated in the legend improved GST-cLD binding.

**Fluorescence Anisotropy**

Ire1-cLD$^{W426A}$ binding to FAM-labeled peptides was measured by the change in fluorescence anisotropy on a Spectramax-M5 plate reader with $\lambda_{ex}$ = 485 nm and $\lambda_{em}$ = 525 nm with increasing concentrations of Ire1-cLD$^{W426A}$. The reactions were measured in a 20 μL volume in 384-well, black flat-bottomed plates after incubation for 30 min. Anisotropy (r) values are calculated as follows: $[(I_{para}-I_{perp})/ (I_{para}+(2* I_{perp}))]$, where $I_{para}$ and $I_{perp}$ are the intensities when the excitation and emission polarizers are parallel and perpendicular to each other. $K_{1/2}$ were calculated by fitting the data to the following equation using Kaleidagraph $r_{free}+(r_{bound}-r_{free}) * (r^n/(r^n+K_{1/2}^n))$, where r is the observed total anisotropy, n is the Hill coefficient, and $K_{1/2}$ is the concentration of Ire1 where 50% of the peptide is bound. $r_{free}$ was set to the anisotropy of the peptide with no protein present, and $r_{bound}$ was set to the anisotropy with the highest concentration of cLD$^{W426A}$. In cases where the maximum anisotropy was not reached, the maximum was set to account for the same polarization change observed for FAM-ΔEspP (Fig. 3A) or cLD$^{W426A}$ (Fig. 3C).

**Sedimentation Velocity and Equilibrium**

Sedimentation velocity experiments were carried out on a Beckman XL-A analytical centrifuge at 40,000xg at 20ºC measuring the absorbance at 280 nm. All samples equilibrated for 30 minutes prior to centrifugation. The reaction volumes were 150 μL of Ire1 cLD (7 μM WT or 9 μM W426A) in Minimal Reaction Buffer (see above). ΔEspP peptide with a solubility tag (C-terminal AAKKKK) was added to 50 μM (WT) and 20 μM (W426A). A total of 75 scans were collected and sedimentation traces were analyzed in Ultrascan 9.8 using C(s) analysis.

Sedimentation equilibrium experiments were carried out on the same instrument, with three different concentrations of Ire1 cLD$^{W426A}$ (4.5, 9, and 13.5 μM) at 3 different speeds (10,000, 14,000, and 20,000xg). Equilibrium was reached after 12 hours. The sedimentation traces were analyzed with Sedfit and Sedphat software, using a monomer model to fit the average molecular weight and a monomer-dimer model to fit the $K_D$ for Ire1 cLD$^{W426A}$ association. For samples with peptide, ΔEspP peptide with a solubility tag (C-terminal AAKKKK) was added at a 2:1 molar ratio.

**Figures and Legends**

**Figure S1.** In the X-ray crystal structure of *S. cerevisiae* Ire1 lumenal domain (PDB: 2BE1), the Ire1 cLD monomer (green and purple) forms symmetric dimers (ex. ribbon model) through Interface 1 to create the putative peptide-binding groove. Within the asymmetric unit cell, these symmetric dimers are connected into an oligomer through Interface 2 (*1*). The amino acid mutated to block oligomerization (W426A) through Interface 2 is colored in red.

Figure S1

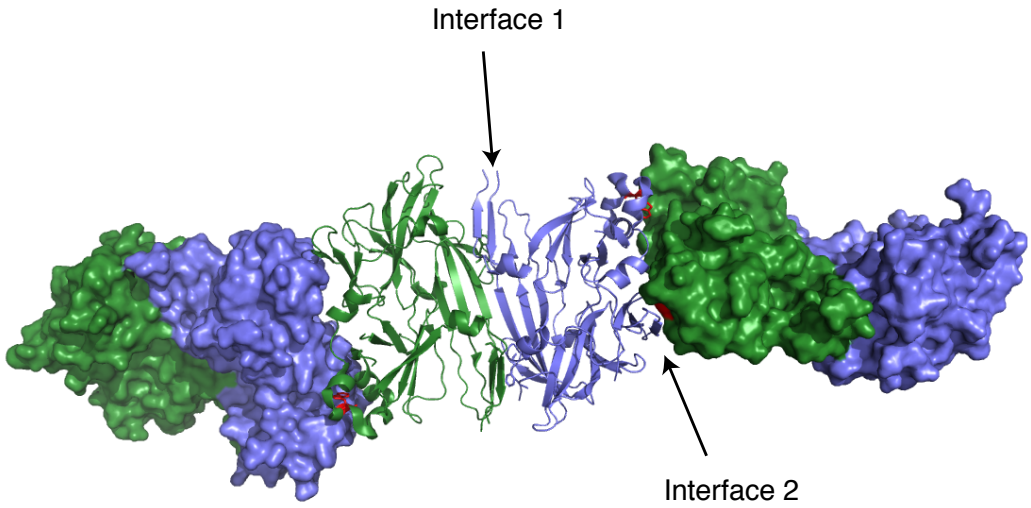


**Figure S2.** The amino acid composition of CPY* was compared to the composition of regions that bound Ire1 cLD WT (the binding cutoff was 0.154 on Fig 2B, which includes 35% of CPY*'s amino acids). Each amino acid was given a binding score that represents the change in its representation amongst the amino acids in CPY* versus Ire1 cLD binding peptides. For example, of the 190 amino acids with an average spot intensity over 0.154, there were 8 arginines. There are 12 arginines in CPY*, which is 544 amino acids in length. The binding score is: (8/190)-(12/544)=2.00. Asterisks indicate where the hypergeometric distribution is less than 0.02.

Figure S2

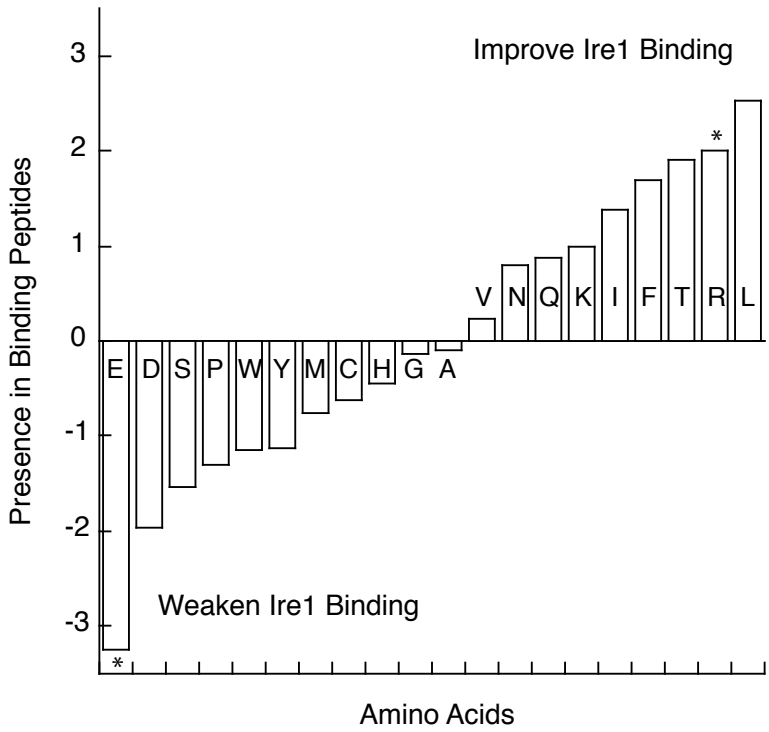

Improve Ire1 Binding
Weaken Ire1 Binding
Presence in Binding Peptides
Amino Acids
3
2
1
0
-1
-2
-3
E D S P W Y M C H G A V N Q K I F T R L
*
*

**Figure S3.** Each amino acid in a binding peptide derived from peptide F17 on the CPY* tiling array (CNNAQLAPYQRTGRNV) was systematically substituted for every other amino acid. Mutated peptides were synthesized on a peptide array and probed with GST-cLD. The change in binding intensity caused by the substitution was normalized to the intensity of the wild type peptide (set at 100%). Substitutions to basic residues are in blue and substitutions to acidic residues are in red. Arrows highlight the basic residues in the F17 peptide.

Figure S3

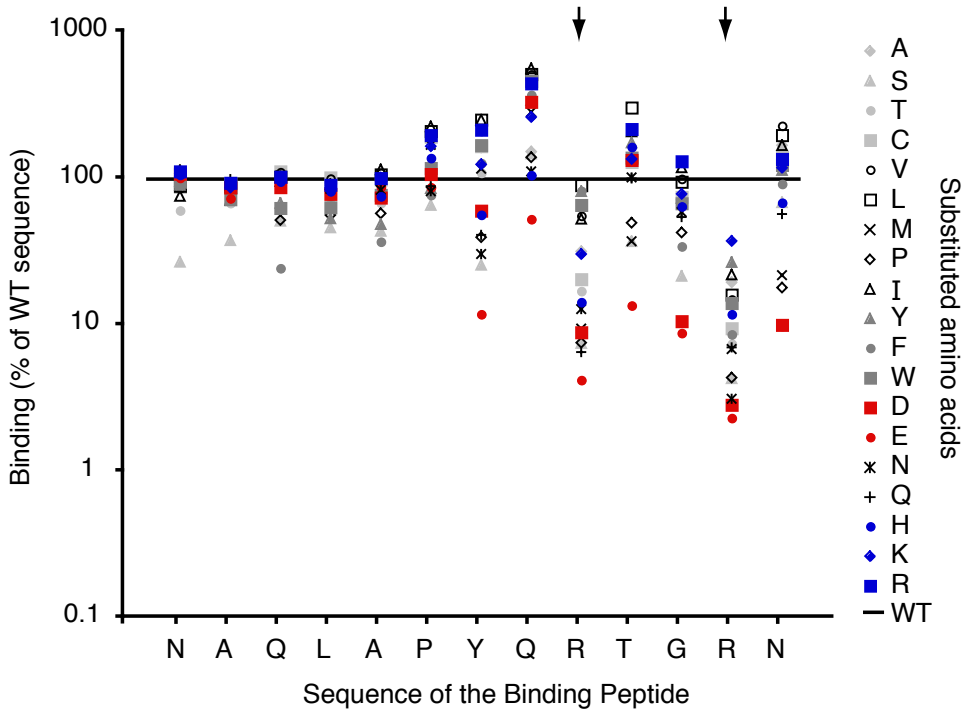

1000
100
10
1
0.1
Binding (% of WT sequence)
N A Q L A P Y Q R T G R N
Sequence of the Binding Peptide
Substituted amino acids
A
S
T
C
V
L
M
P
I
Y
F
W
D
E
N
Q
H
K
R
WT

**Figure S4.** A. Ire1-FLAG was immunoprecipitated from yeast expressing fragments of CPY* (pink, which contains the peptide F17: aa 341-373, purple: aa 374-436, blue: aa 437-468) attached to FKBP (gray circle) as a scaffold protein. The expression of the fragments was induced by 2 hours in 2% galactose. B. The UPR induction caused by the expression of each CPY* fragment-FKBP fusion was monitored with the splicing reporter after 3 hours in 2% galactose. Error bars are SD; N=4.

Figure S4

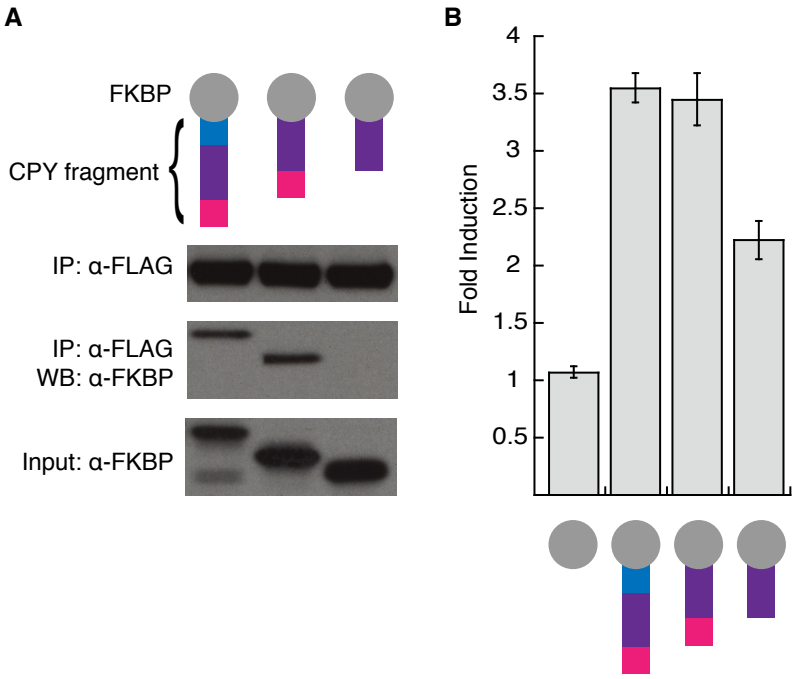

A
FKBP
CPY fragment
IP: α-FLAG
IP: α-FLAG
WB: α-FKBP
Input: α-FKBP
B
Fold Induction
4
3.5
3
2.5
2
1.5
1
0.5

**Figure S5.**
Amino acids within ΔEspP were mutated two at a time to either glutamic acid or alanine and probed with GST-Ire1 cLD$^{W426A}$ as in Fig. 2A. The effect of each mutation was quantified by comparing the intensity of the spot to WT (at the bottom, set to 100%). The addition of acidic residues or removal of basic residues attenuates GST-Ire1 cLD$^{W426A}$ binding.

Figure S5.

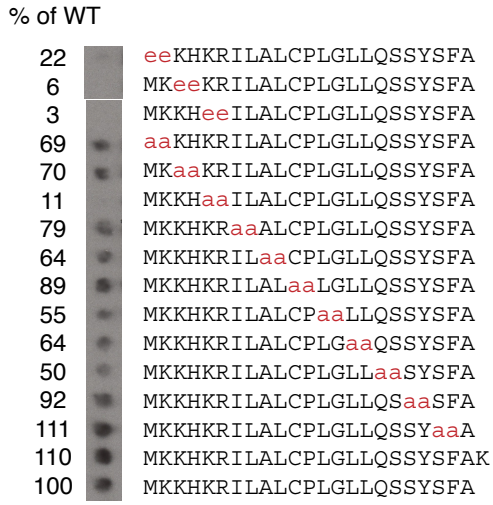
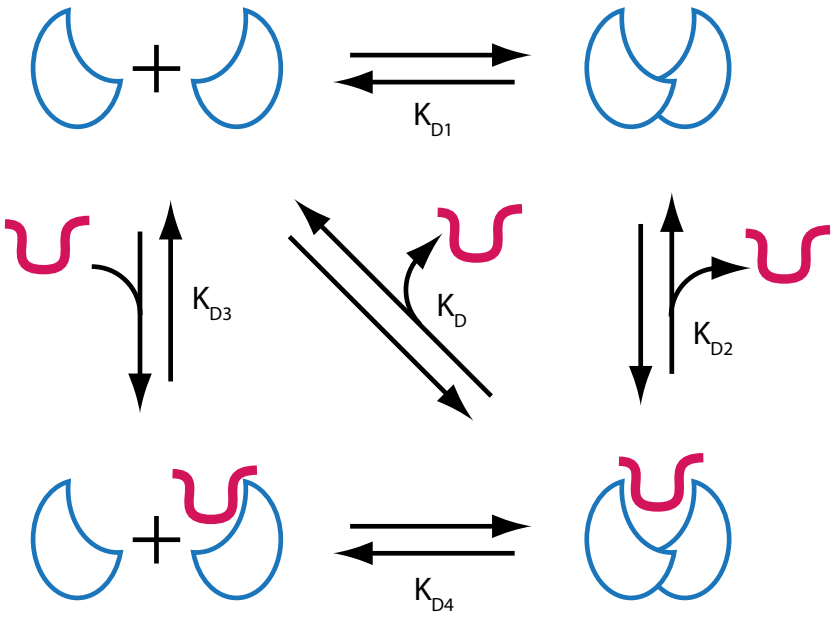

% of WT
22 eeKHKRILALCPLGLLQSSYSFA
6 MKeeKRILALCPLGLLQSSYSFA
3 MKKHeeILALCPLGLLQSSYSFA
69 aaKHKRILALCPLGLLQSSYSFA
70 MKaaKRILALCPLGLLQSSYSFA
11 MKKHaaILALCPLGLLQSSYSFA
79 MKKHKRaaALCPLGLLQSSYSFA
64 MKKHKRILaaCPLGLLQSSYSFA
89 MKKHKRILALaaLGLLQSSYSFA
55 MKKHKRILALCPaaLLQSSYSFA
64 MKKHKRILALCPLGaaQSSYSFA
50 MKKHKRILALCPLGLLaaSYSFA
92 MKKHKRILALCPLGLLQSaaSFA
111 MKKHKRILALCPLGLLQSSYaaA
110 MKKHKRILALCPLGLLQSSYSFAK
100 MKKHKRILALCPLGLLQSSYSFA

**Figure S6.** This figure models the equilibriums of Ire1 cLD (blue) binding to peptide (pink) and Ire1 dimerization in the simplified case of the $cLD^{W426A}$ mutant. $K_{D1}$, which is for Ire1 dimerization in the absence of peptide, was measured by sedimentation equilibrium (Fig. 4C and S8) and is 8.2 μM for Ire1 $cLD^{W426A}$. From velocity sedimentation experiments, we know that there is a minimal population of Ire1 monomer bound to peptide in solution (absence of a monomer peak in Fig. 4B), which suggests that during our fluorescence anisotropy experiments we are measuring the difference between free peptide and peptide bound by an Ire1 dimer, or the reaction represented by $K_D$. Assuming a cooperative binding reaction with a Hill coefficient of 2, the $K_D = (K_{1/2})^2$. The observed cooperativity of Ire1 binding in the fluorescence anisotropy assay is 1.2 (FAM-ΔEspP) and 1.4 (F17-FAM) (Figure 3A), although higher cooperativity may not be apparent because of the low signal to noise ratio at lower concentrations if $cLD^{W426A}$. However, the $K_{1/2}$ is similar when modeled with a Hill coefficient of 1 or 2 (range: 0.70-0.75 μM). Using these assumptions, we can calculate $K_{D2}$, which is the affinity of an Ire1 dimer for peptide: $K_{D2}=K_D /K_{D1}= (0.75\ \mu M)^2 /(8.2\ \mu M)= 0.068\ \mu M$.

Figure S6




$$K_{D1} = \frac{[\,]^2}{[\,]} \qquad K_{D2} = \frac{[\,][\,]}{[\,]}$$

$$K_{D3} = \frac{[\,][\,]}{[\,]} \qquad K_{D4} = \frac{[\,][\,]}{[\,]}$$

$$K_D = \frac{[\,]^2[\,]}{[\,]}$$

$$K_{D1}K_{D2} = K_{D3}K_{D4} = K_D$$

$$\text{Fraction of Ire1 bound} = \frac{[\,]^2}{[\,]^2 + K_D} \approx \frac{[\,]^2}{[\,]^2 + (K_{1/2})^2}$$

**Figure S7.** Serial dilutions of CRY1 Δ*ire1*::KanMX6 strains containing CEN-ARS plasmids expressing either no Ire1, WT Ire1, or Ire1 ΔMFY under its own promoter. Ire1 ΔMFY exhibits a growth defect on plates containing tunicamycin to induce ER stress.

Figure S7

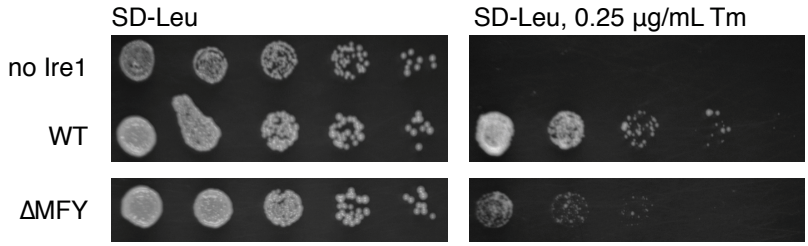
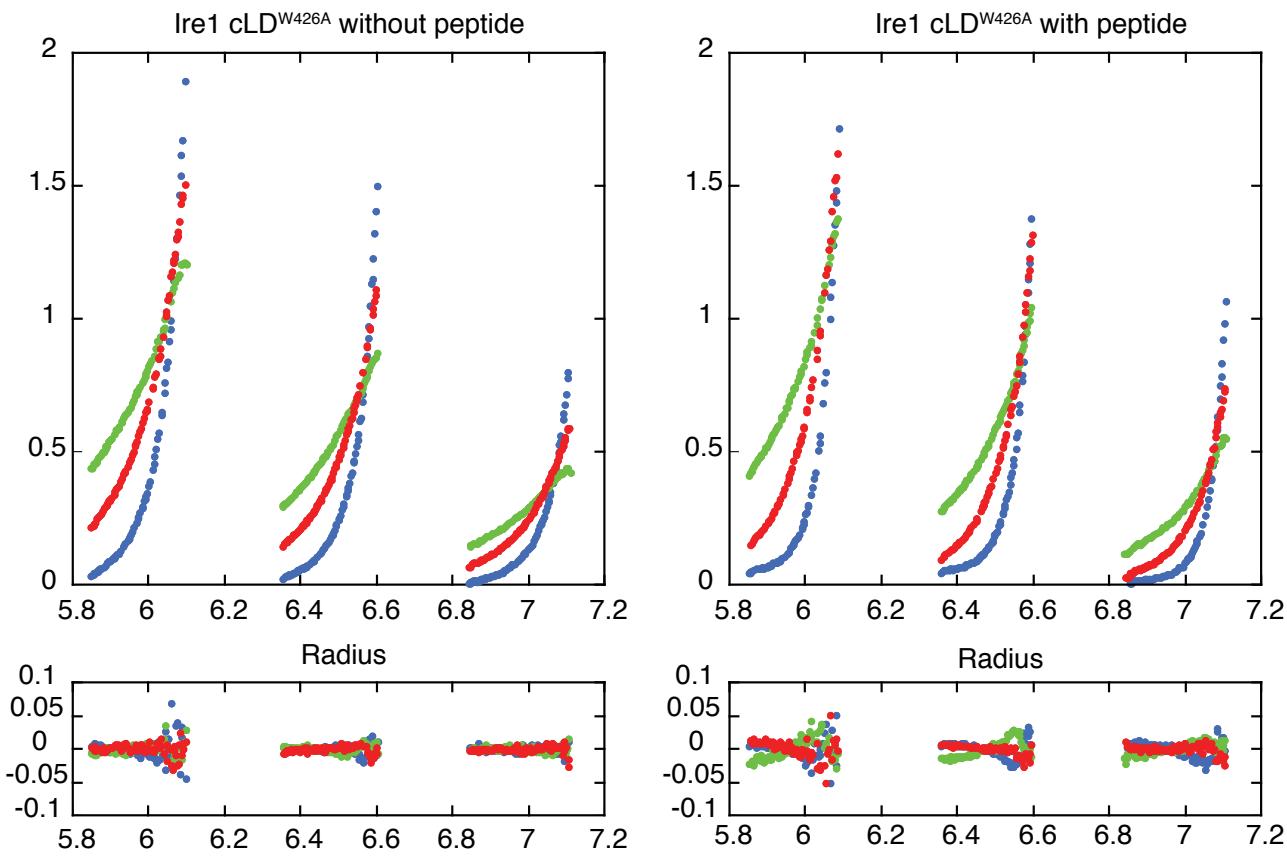


**Figure S8.** Sedimentation equilibrium profiles of Ire1 cLD$^{W426A}$ at three different concentrations (4.5, 9, and 13.5 μM) were achieved at three different speeds (10,000xg, 14,000xg, and 20,000xg) with and without a 2:1 molar ratio of ΔEspP. The residuals of fitting a monomer-dimer equilibrium using SedFit software are shown below the profiles. The average molecular weight increases from 62 kDa to 88 kDa upon addition of peptide and the $K_D$ of Ire1-Ire1 dimerization without peptide is 8.2 μM.

Figure S8


Ire1 cLD$^{W426A}$ without peptide
Ire1 cLD$^{W426A}$ with peptide
Radius
Radius
2
1.5
1
0.5
0
0.1
0.05
0
-0.05
-0.1
5.8
6
6.2
6.4
6.6
6.8
7
7.2