Supplementary Data

Supplementary Materials and Methods

Plasmid construction and mutagenesis

All PCRs were performed using the Phusion High-Fidelity PCR Master Mix (NEB). The *trxA* gene was amplified from *Escherichia coli* MRE600 chromosomal DNA and cloned into vector pET19B (Novagen) using *Nde*1 and *Xho*1. The *ychF* gene was subcloned from pET22b-YchF (6) into plasmid pBad using *Hind*III and *Xba*I. By inverse PCR, all six or individual cysteine residues of YchF were mutated to serine (Supplementary Table S1). Inverse PCR of pBad-YchF was used to generate amber stop codon mutants of YchF.

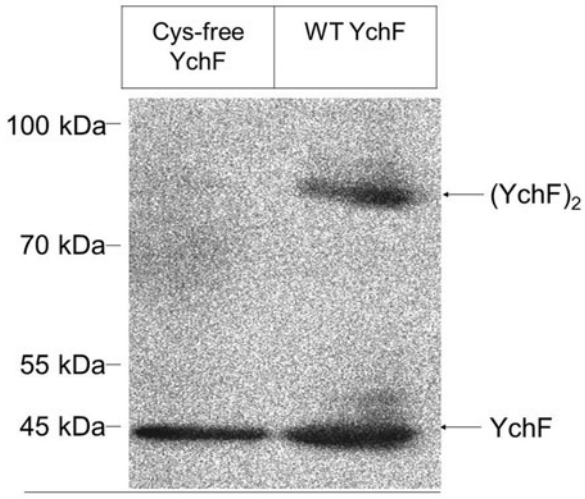
Identification of disulfide-linked peptides by MS

MS/MS data files were converted into the mzXML format using the ProteoWizard software (version 3.0.6965) (1). Disulfide-linked peptides were identified using the xQuest/xProphet software (version 2.1.1) (3, 5). Searches were performed against the amino acid sequences of the recombinant protein as well as the contaminants, trypsin and keratin II (UniProt accessions, P00761 and P35908). Reversed sequences were created using the xdecoy tool included in the xQuest pipeline. Enzyme specificity was set to trypsin with up to one missed cleavage. Oxidation of methionine residues was considered as variable modification. The MS^1 mass tolerance was set to 5 ppm and MS^2 tolerances to 0.4 Da for cross-linker-containing ions and 0.3 Da for common ions. Mass shifts were set to –2.0156 for peptides cross-linked by disulfides between two cysteine residues and to 57.02146 and 125.0477 for monolinked peptides, accounting for peptides modified at a single cysteine residue by iodoacetamide and NEM, respectively. Settings for unlabeled cross-linkers were used and default values for all other parameters. Identifications were filtered, applying a false discovery rate (FDR) of less than 1% computed using xProphet (version 2.5.1) and a minimum delta score of ≤0.95.

Relative quantification of confident hits (FDR <0.01) was done using Skyline (version 3.1.0) (4). To extract the ion chromatograms of identified disulfide-linked peptides, they were regarded as one linear peptide with the second peptide as a variable modification at the cysteine residue. These variable modifications were defined by their chemical formulas and masses and added to the list of modifications in the peptide settings in Skyline *via* a*.skys file. Peptide search results were imported into Skyline by mimicking parameter and result formats of the MaxQuant program (2). Data import was performed with the following filter transition settings: precursor charges, +2 to +6; ion charges, +1 to +3, product ions, from the first to last ion; and the following full-scan transition settings: MS^1 filtering, four isotope peaks included; resolving power, 60.000 at *m/z* 400; retention time filtering, including all matching scans. The resulting extracted ion chromatograms were manually inspected for proper peak picking. Peak boundaries were manually adjusted if necessary. Total peak area (*i.e.*, peak area integrated over the four isotopic MS^1 peaks) was exported for each modified sequence and each sample and peak areas of identical Cys-Cys links were summed up.

Supplementary Table S1. Oligonucleotide Primer Used for Site-Directed Mutagenesis

Name	*Sequence*	*Function*
YchF_168C>S_For*	5′-gaa aaa tcc ctg ccc ca-3′	Inverse PCR for YchF, Cys 168-> Serin, forward
YchF_168C>S_Rev	5′-cag gac cgc cag ctc-3	Inverse PCR for YchF, Cys 168-> Serin, reverse
YchF_140C>S_For*	5′-gac acc tcc gaa cgt gcg-3′	Inverse PCR for YchF, Cys 140-> Serin, forward
YchF_140C>S_Rev	5′-gag gtc tgc cag cgc cag-3′	Inverse PCR for YchF, Cys 140-> Serin, reverse
YchF_237C-> S_For*	5′-gtt ccg gtt tct gct gct gtt-3′	Inverse PCR for YchF, Cys 237-> Serin, forward
YchF_237C-> S_Rev	5′-cac aac aga acc ttc ttt cgc-3′	Inverse PCR for YchF, Cys 237-> Serin, reverse
YchF_106C-> S_For*	5′-gtt gtt cgc tcc ttt gaa aat-3′	Inverse PCR for YchF, Cys 106-> Serin, forward
YchF_106C-> S_Rev	5′-gtg acc gat cgc ttc ggt-3′	Inverse PCR for YchF, Cys 106-> Serin, reverse
YchFNeuCys-> Ser5for	5′-ggt ttg ccc aac gtc g-3′	Inverse PCR for YchF, Cys 5-> Serin, forward
YchFNeuCys-> Ser5*rev	5′-gac gat acc gga ttt gaa tcc-3′	Inverse PCR for YchF, Cys 5-> Serin, reverse
YchFNeuCys-> Ser35for	5′-ccg aac aca ggc gtc gta-3′	Inverse PCR for YchF, Cys 35-> Serin, forward
YchFNeuCys-> Ser35*rev	5′-ctc aat ggt gga gaa tgg aaa-3′	Inverse PCR for YchF, Cys 35-> Serin, reverse
TrxAChromNde1for	5′-gtg gag tta cat atg agc gat aaa att att cac ct-3′	TrxA for pet19B, forward, Nde1
TrxAChromXho1rev	5′-gaa ctc gag tta cgc cag gtt agc gt-3′	TrxA for pet19B, reverse, Xho1



SUPPLEMENTARY FIG. S1. Purification under denaturing conditions does not prevent dimer formation in wild-type YchF. Wild-type (Wt) YchF and a cysteine-free YchF derivative were purified under denaturing conditions in the presence of 6M urea. After refolding, samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis under nonreducing conditions. After Western transfer, the samples were probed with α-YchF antibodies. Indicated are the YchF monomer and dimer.

Supplementary References

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