SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Mass spectrometry – Recombinant BiP protein was oxidized with GSSG as described in the Experimental Procedures. Protein was separated by non-reducing SDS-PAGE, the gel was stained with SYPRO Ruby, and the two major visible bands at ~72 kDa and ~150 kDa were excised from the gel. Gel slices were cut into ~1 mm cubes and subjected to in-gel digestion with chymotrypsin (Roche) followed by extraction of the chymotryptic peptides as reported previously (1). The excised gel pieces were washed consecutively in distilled water, 100 mM ammonium bicarbonate (Ambic)/acetonitrile (1:1), and acetonitrile (ACN). The gel pieces were directly alkylated with 55 mM iodoacetamide in 100 mM Ambic at room temperature in the dark for 60 min without the traditional reduction step. After repeating the wash steps described above, the gel slices were dried and rehydrated with chymotrypsin (20 ng/µl) in 50 mM Ambic, 10% ACN at 37 °C for 16 h. The digested peptides were extracted twice with 50% ACN, 5% formic acid (FA) and once with 90% ACN, 5% FA. Extracts from each sample were combined and lyophilized.

The in-gel tryptic digests were reconstituted in 0.5% FA for nanoLC-ESI-MS/MS analysis, which was carried out by an Orbitrap Elite mass spectrometer (Thermo-Fisher Scientific, San Jose, CA) equipped with a "CorConneX" nano ion source device (CorSolutions LLC, Ithaca, NY). The Orbitrap was interfaced with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA). A portion of the gel extracted peptide samples were injected onto a PepMap C18 trap column-nano Viper (5 µm, 100 µm x 2 cm, Thermo) at 20 µl/min flow rate for on-line desalting and then separated on a PepMap C18 RP nano column (3 µm, 75 µm x 25 cm, Thermo) which was installed in the nano device with a 10-µm spray emitter (NewObjective, Woburn, MA). The Orbitrap calibration and nanoLC-MS/MS operation were as described previously (2). The peptides were eluted with a 90 min gradient of 5% to 38% ACN in 0.1% FA at a flow rate of 300 nl/min, followed by a 5-min ramp to 95% ACN, 0.1% FA and a 7-min hold at 95% ACN, 0.1% FA. The Orbitrap Elite was operated in positive ion mode with the nano spray voltage set at 1.5 kV and a source temperature of 250 °C.

The instrument was operated in parallel data-dependent acquisition (DDA) under FT-IT mode using a FT mass analyzer for one MS survey scan from m/z 375 to 1800 with a resolving power of 120,000 (fwhm at m/z 400) followed by MS/MS scans on the top 15 most intensive peaks with multiple charged ions above a threshold ion count of 10,000 in the FT mass analyzer. External calibration using Ultramark 1621 for both FT mass analyzer and IT mass analyzer was performed. Dynamic exclusion parameters and normalized collisional energy were set the same as described previously (2, 3). All data were acquired under Xcalibur 2.2 operation software (Thermo-Fisher Scientific).

Peptides with potential post-translational modification of the BiP Cys residue identified in the ~72 kD gel band were confirmed by injection of the above gel-extracted digests in an Orbitrap FusionTM TribridTM (Thermo-Fisher Scientific, San Jose, CA) mass spectrometer equipped with a nanospray Flex Ion Source, and coupled with a Dionex UltiMate3000RSLCnano system (Thermo, Sunnyvale, CA) with a 90 min gradient similarly as describe above. The Orbitrap Fusion was operated in positive ion mode with the spray voltage set at 1.6 kV and source temperature at 275°C. In DDA analysis, the instrument was operated using the FT mass analyzer in MS scan mode to select precursor ions followed by 3 sec "Top Speed" data-dependent CID (2 charged ions only) and ETD (≥ 3 charged ions) ion trap MS/MS scans at 1.6 m/z quadrupole isolation for precursor peptides above a threshold ion count of 10,000. The normalized collision energy was 30% for CID, and 150 ms for ETD reaction time and 200 ms for maximal ETD reagent injection time were used for ETD analysis. MS survey scans at a resolving power of 120,000 (fwhm at m/z 200), for the mass range of m/z 375-1575. Dynamic exclusion parameters were set at a repeat count of 1 with a 20 sec repeat duration, an exclusion list size of 500, 40 sec of exclusion duration with ± 10 ppm exclusion mass width. The activation time was 10 msec for

CID analysis. All data were acquired under Xcalibur 3.0 operation software (Thermo-Fisher Scientific).

The DDA raw files for CID MS/MS only were subjected to database searches using Proteome Discoverer (PD) 1.4 software (Thermo Fisher Scientific, Bremen, Germany) with the Sequest HT algorithm. The CID-ETD raw data file was searched using PD 2.1 software. The database search was conducted against a customized *E coli* database containing 4,941 entries plus the recombinant yeast BiP protein with two-missed chymotrypsin cleavage sites allowed. The peptide precursor tolerance was set to 10 ppm and fragment ion tolerance was set to 0.8 Da, and variable modification of the cysteine (glutathione, carbidomethyl, and NEM), methionine oxidation, N-terminal acetylation and deamidation of asparagine or glutamine were set. Only high confidence peptides defined by Sequest HT with a 1% fixed false-discovery rate by Percolator were considered for the peptide identification. The final protein identifications contained protein groups that were filtered with at least 2 peptides per protein. All MS/MS spectra for identified Cys-modified peptides from initial database searching were manually inspected and validated using Xcalibur 2.2.

SUPPLEMENTAL REFERENCES

1. Yang, Y., Thannhauser, T. W., Li, L., and Zhang, S. (2007) Development of an integrated approach for evaluation of 2-D gel image analysis: impact of multiple proteins in single spots on comparative proteomics in conventional 2-D gel/MALDI workflow. *Electrophoresis*, **28**, 2080-2094
2. Yang Y, Qiang X, Owsiany K, Zhang S, Thannhauser T.W., and Li L. (2011) Evaluation of different multidimensional LC-MS/MS pipelines for isobaric tags for relative and absolute quantitation (iTRAQ)-based proteomic analysis of potato tubers in response to cold storage. *J Proteome Res.* **10**, 4647-60
3. Hochrainer, K., Racchumi, G., Zhang, S., Iadecola, C., and Anrather, J. (2012) Monoubiquitnation of nuclear RelA negatively regulates NF-kB activity independent of proteasomal degradation. *Cell Mol Life Sci*. **69**, 2057-73

Table S1: Protein composition of the ~150 kD band observed after oxidation of recombinant BiP with GSSG. Recombinant BiP, purified from *E. coli*, was oxidized with GSSG as described in the Experimental Procedures. Protein was separated by non-reducing SDS-PAGE, the gel was stained with SYPRO Ruby, and the ~150 kD band was excised from the gel. Excised protein was subject to in-gel digestion under non-reducing conditions with chymotrypsin, alkylated with iodoacetamide, and analyzed by nanoLC-ESI-MS/MS. Peptides were identified after searching a customized database for matching recombinant BiP and/or *E. coli* peptides. Of note, underlined proteins contain no cysteines, and these proteins are not expected to form an interaction with recombinant BiP that is reversible with a reducing agent.

Accession #	Description	Score	Coverage	# Unique Peptides	MW [kDa]
P00001	Recombinant *S. cerevisiae* BiP	1129.57	73.04	51	72.3
16128896	*E. coli* OmpF	111.59	51.38	22	39.3
16129047	*E. coli* Rne	59.91	22.53	12	118.1
16128701	*E. coli* SucA	12.34	7.18	5	105.0
16131810	*E. coli* TufB	11.67	15.74	3	43.3
16131845	*E. coli* MetH	10.92	3.83	3	135.9
16128980	*E. coli* PutA	8.85	4.02	4	143.7
2851542	*E. coli* FdoG	8.27	5.61	4	112.4
43107	*E. coli* unnamed protein product	7.34	3.84	1	54.0
15799694	*E. coli* DnaK	6.62	6.27	2	69.1
16130190	*E. coli* ArnA	6.60	4.39	3	74.2
16128633	*E. coli* HscC	6.31	2.16	1	61.9
16130169	*E. coli* NrdA	5.30	1.31	1	85.7
3183546	*E. coli* NuoG	3.46	1.54	1	100.2
15804555	*E. coli* ArgB	3.17	12.79	1	27.1
90111620	*E. coli* EnvC	3.12	6.68	1	46.6
16128857	*E. coli* FtsK	2.91	1.20	1	146.6
15800433	*E. coli* SucD	2.76	4.15	1	29.8
16128644	*E. coli* MiaB	2.09	2.74	1	53.6
16128698	*E. coli* SdhA	1.80	1.53	1	64.4
15803542	*E. coli* putative hydrogenase subunit	0.00	3.76	1	39.6
16129139	*E. coli* MinC	0.00	10.82	1	24.8
16131597	*E. coli* GlmS	0.00	3.78	1	66.9
49176132	*E. coli* YdhC	0.00	1.99	1	43.3
16129108	*E. coli* CohE	0.00	11.61	1	25.1