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Supplementary Materials for

Hyperplexing: A Method for Higher-Order Multiplexed Quantitative Proteomics Provides a Map of the Dynamic Response to Rapamycin in Yeast

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This PDF file includes:

Other Supplementary Material for this manuscript includes the following:
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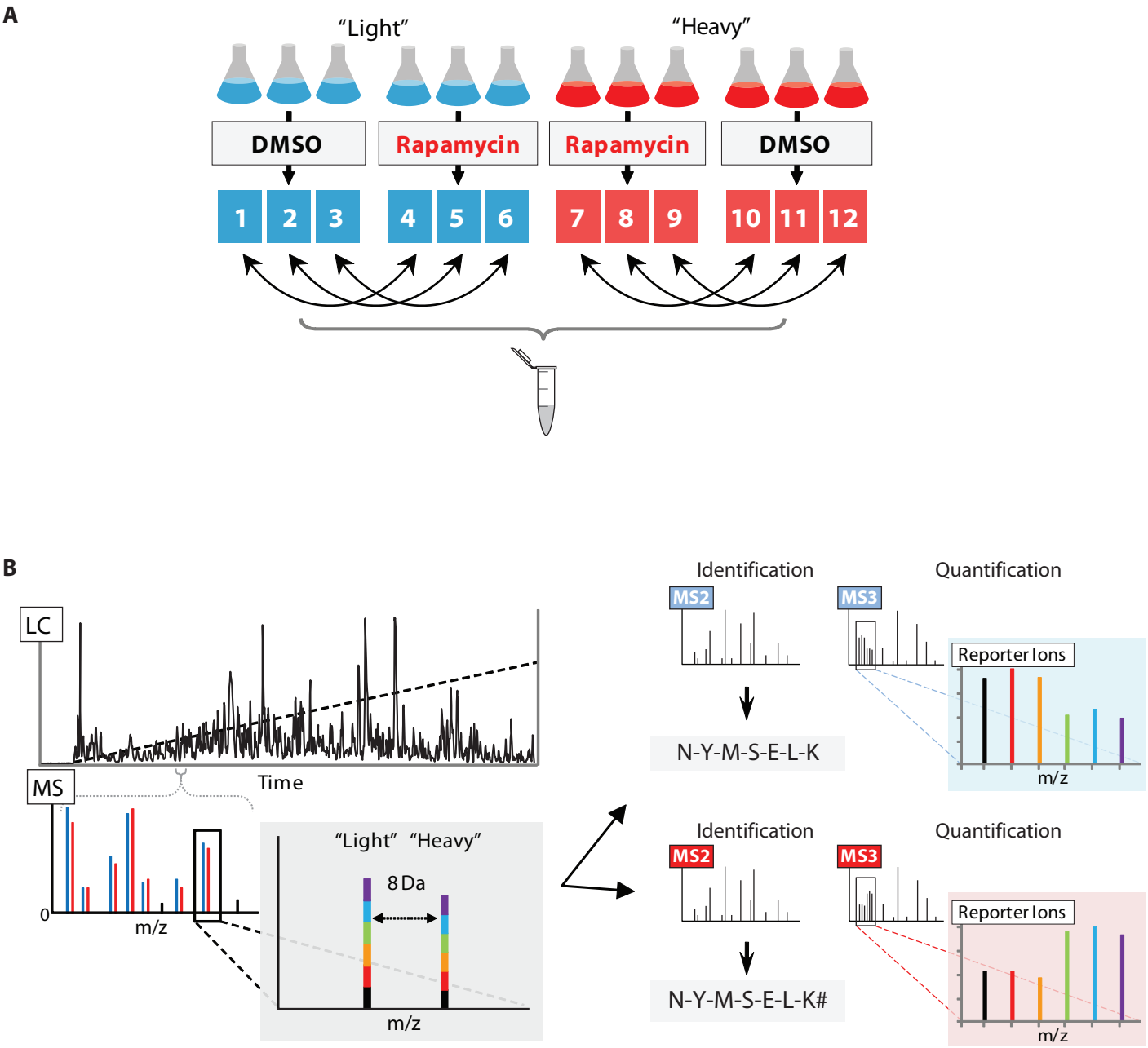

Fig. S1. Proteomic analysis of rapamycin-stimulated yeast by hyperplexing. (**A**) Schematic representation of the "2 X 6", fixed point assay. 12 yeast cultures were grown in synthetic complete media containing either naturally occurring lysine, or $^{13}C_6{}^{15}N_2$-lysine ("Heavy"). Cells were treated with either DMSO or 200 nM rapamycin for 60 min. Equal amounts of lysC-digested extracts were labeled with six-plex TMT reagents and combined. (**B**) Analysis of hyperplexed samples by an MS3 method (*13*). As shown for a "2 X 6" experiment, same sequence peptides with differentially labeled lysines can be differentiated in a MS1 scan. Each species was selected for fragmentation by CID and MS2 in the ion trap for peptide identification. The largest peak from the MS2 scan was isolated and fragmented by HCD. The fragment ions were detected and quantified in a subsequent MS3 spectrum.

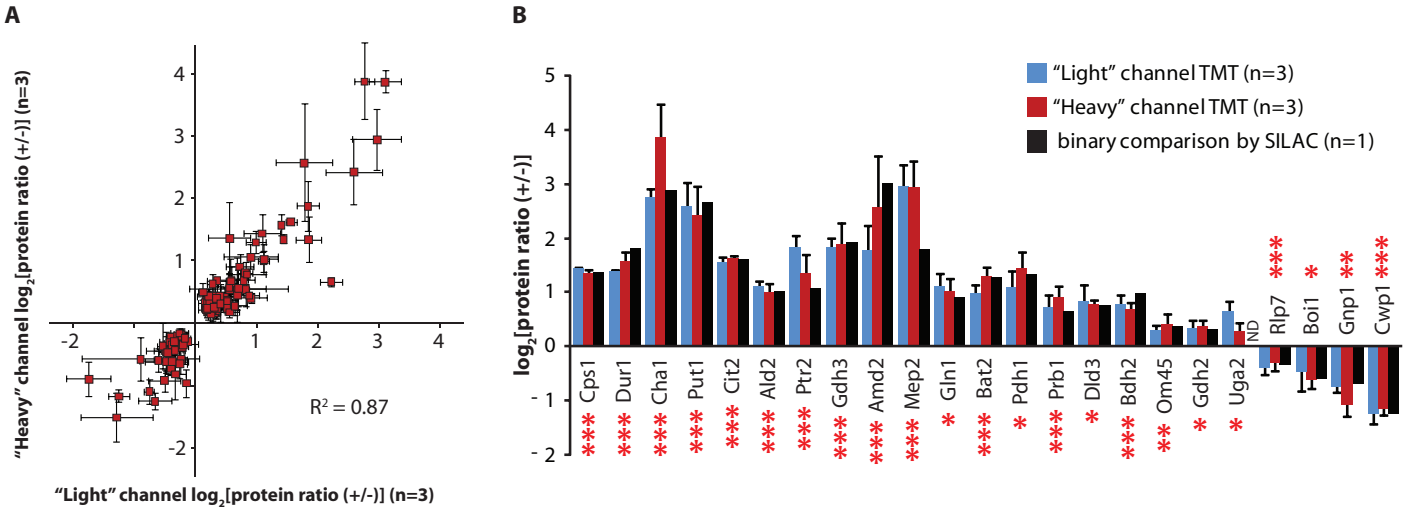

Fig. S2. Quantitative reproducibility and accuracy of the hyperplexing method. (**A**) Correlation of the contribution of each metabolic channel to the 121 rapamycin-regulated proteins. The "light" and "heavy" channel $\log_2$ ratios were plotted separately (n=3 for each). Error bars indicate standard deviation. (**B**) Examples of rapamycin-regulated proteins detected by hyperplexing compared to SILAC-based quantification. We performed a separate binary SILAC experiment comparing rapamycin- and DMSO-treated yeast. Ratios measured from MS1 peaks in the SILAC experiment were compared to regulated proteins quantified by hyperplexing. To highlight reproducibility, the hyperplexed ratios are displayed as two sets of biological triplicates derived from the "light" and "heavy" channels. Error bars represent standard deviation (n=3). ND = not detected. Asterisks indicate three different t-test derived significance value thresholds: * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

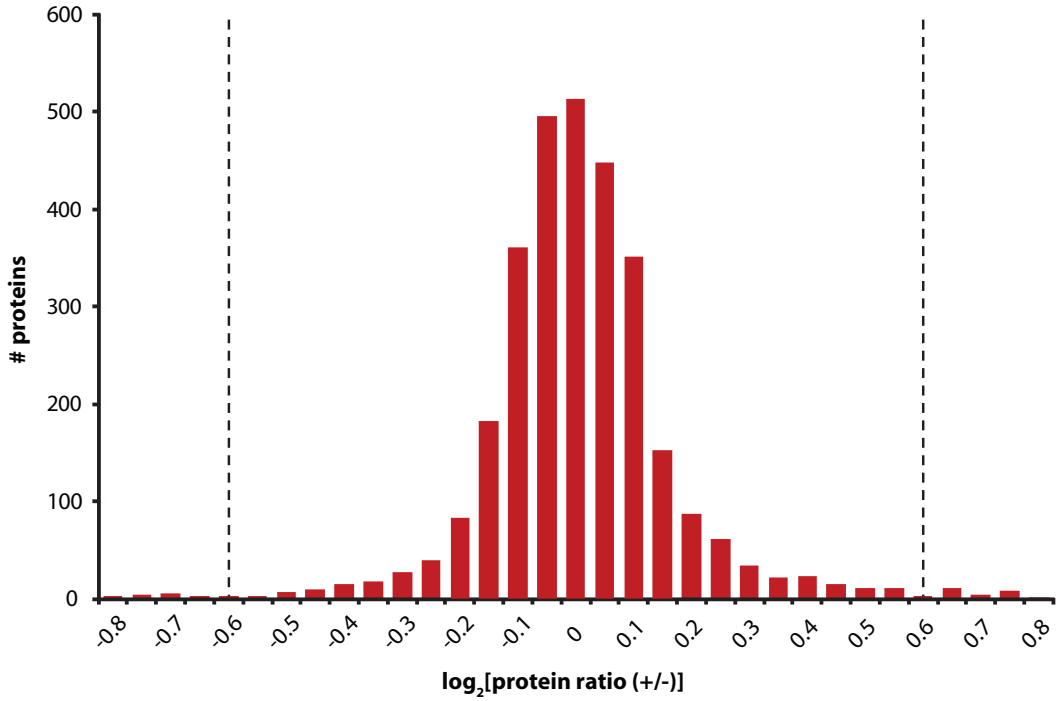

Fig. S3. Protein quantification by SILAC. The distribution of 3073 (1% FDR) $\log_2$ protein abundance ratios measured from cells treated with 200 nM rapamycin (+) or DMSO (-) for 60 min from a single SILAC comparison. (s.d.=0.28). Dotted lines indicate the positions of ± 1.5-fold changes.

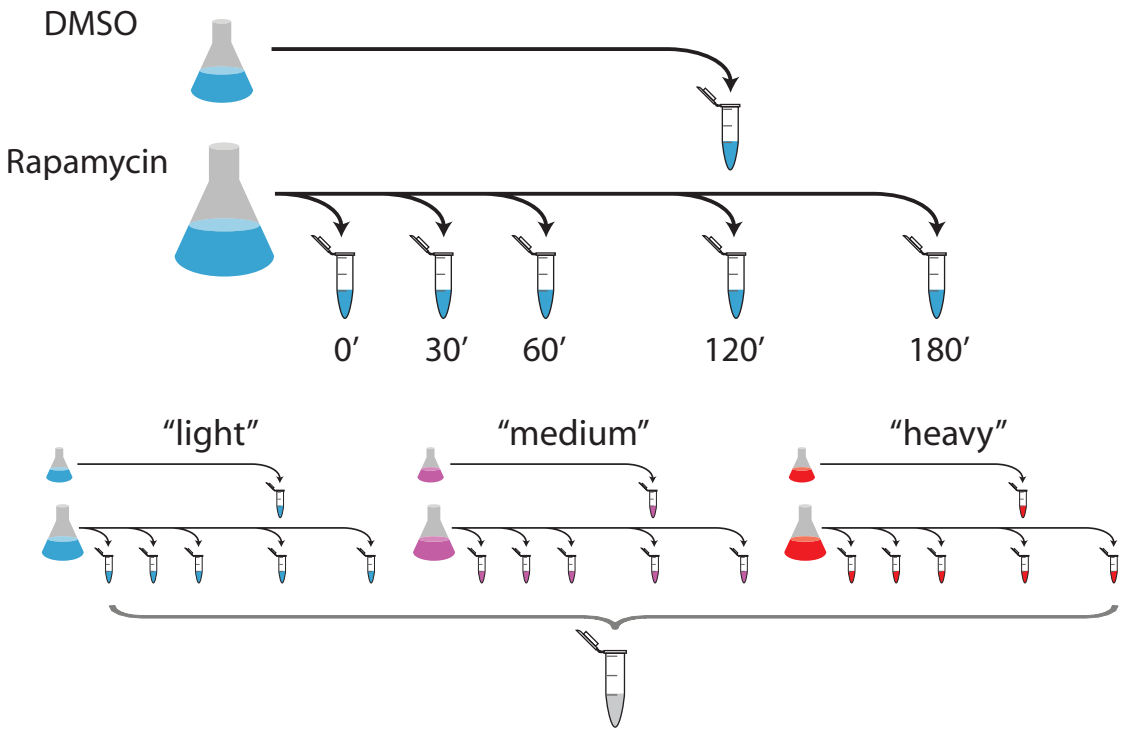

Fig. S4. “3 x 6” hyperplexed analysis of rapamycin-stimulated yeast. The experiment, as depicted for the “light” grown above, was performed in triplicate. Cells were grown to early log phase in “light”, “medium” ($^{13}C_6{}^{15}N_2$, same as the “heavy” in the previous experiment), or “heavy” ($^{13}C_6{}^{15}N_2D_9$-lysine) media and treated with 200 nM rapamycin or DMSO. Samples were taken at the indicated times and equal amounts of digested protein were labeled with six-plex TMT reagents and combined for analysis.

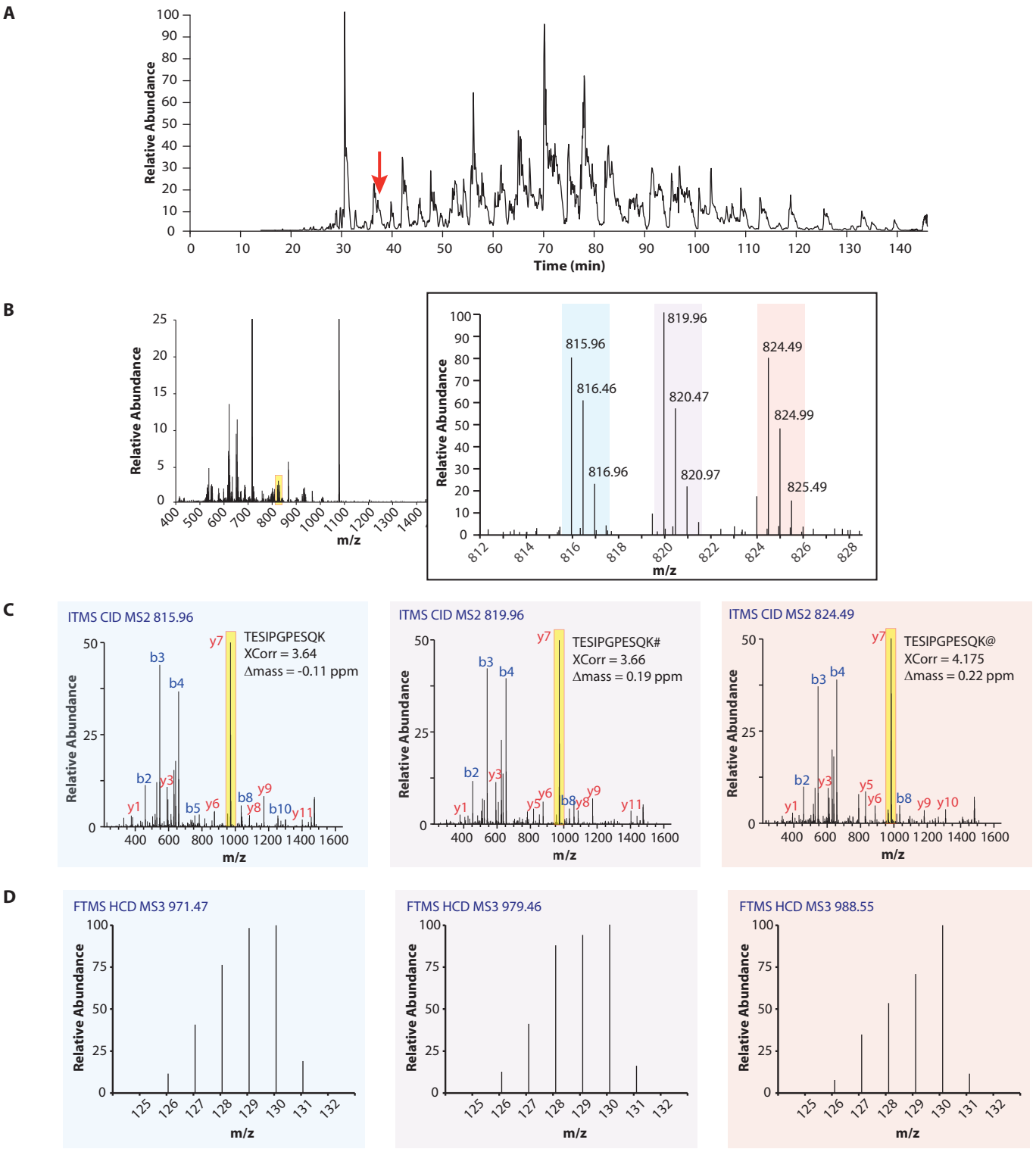

Fig. S5. Workflow for identification and quantification of a representative protein from the "3 x 6" experiment. A single regulated peptide from Uga1, a gamma-aminobutyrate transaminase, is presented as an example. (**A**) Shown is the base peak chromatogram of the LC-MS/MS run for SCX fraction 11. (**B**) A single MS1 scan, collected at 36.7 min (indicated by the arrow in (**A**), is shown with the region near m/z=820 expanded to highlight a triplet of "light", "medium", and "heavy" ions. (**C**) Each ion was selected, fragmented by CID, and detected in the ion trap to produce the shown MS2 spectra. Scans were searched using SEQUEST to provide peptide identification. The highlighted peak in each MS2 spectrum was isolated, fragmented by HCD, and detected and quantified in an MS3 in the orbitrap. (**D**) The low mass range of the MS3 scans containing the TMT reporter ions used for quantification is shown for each. Reporter ion peaks at m/z = 126, 127, 128, 129, 130, and 131 represent samples taken at t = 0 min, 30 min, 60 min, 120 min, 180 min, and the DMSO control respectively.

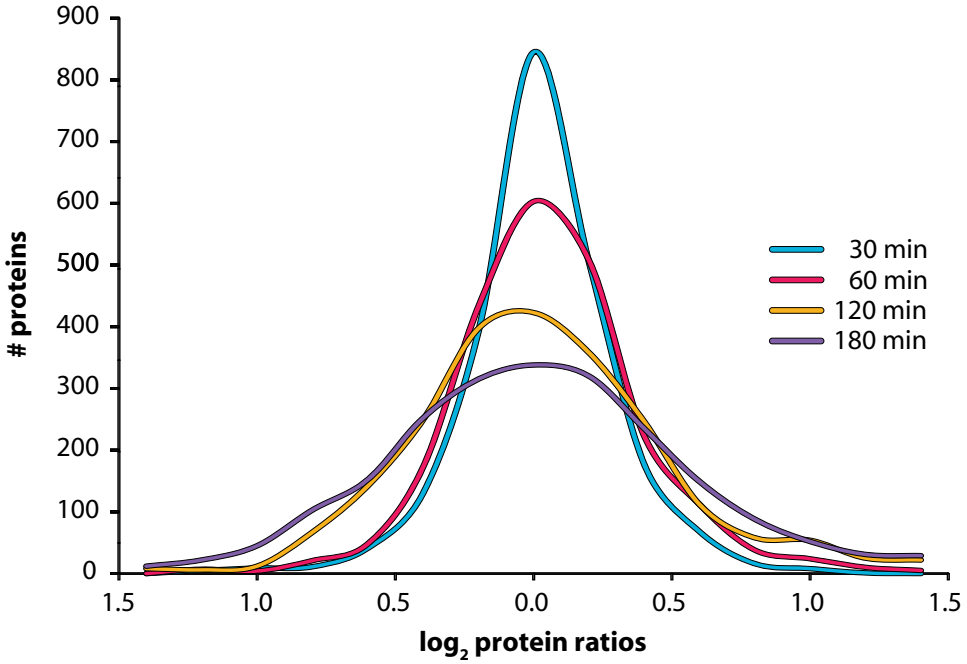

Fig. S6. Delayed response of the yeast proteome to 200 nM rapamycin. The distribution of protein ratios (relative to 0 min) was plotted for each time point after rapamycin stimulation. The same set of 2217 proteins was used in all plots. Bin size = 0.2.