**Materials and Methods**

**Yeast Strains, Media and Plasmids**

All yeast strains used in this study are listed in Table 7. Each APP model contains one or multiple copies of the heterologous gene integrated into the genome under the control of a galactose promoter. The TDP43, αSyn and HTT strains were C-terminally tagged with GFP. Wild type W303 yeast transformed with the vector reported in Table 7 was used as the empty vector (EV) control. For imaging of FUS, wild type W303 yeast was transformed with the pAG426Gal-ccdB-FUS-EYFP vector using the standard LiAc protocol (Gietz and Woods, 2002). All yeast media used in this study are listed in table S7.

**Induction of Protein Overexpression**

Single yeast colonies were inoculated into 5 ml SD-Glu and grown overnight at 30°C under shaking at 180 rpm. The following day cells were transferred to SD-Raf to a final OD600 of 0.01. The overnight SD-Raf cultures were diluted into SD-Gal to a final OD600 of 0.3 for the induction of APP protein overexpression. Cell lysates were collected at 6, 12, 18 and 24 hours after induction of APP overexpression.

**Spotting Assay**

Yeast precultures were grown overnight in 5 ml SD-Glu at 30°C under shaking at 180 rpm. The following day, cells were transferred to SD-Raf to a final OD600 of 0.7. Cells were then serially diluted in sterile water and 5 µl of each dilution was spotted onto solid medium supplemented with the appropriate carbon source (2% glucose or 2% galactose) and left to air dry at room temperature under sterile conditions. Growth was detected after three days of incubation at 30°C and photographed with a digital camera.

**Growth Curves**

The growth of the different APP models and the control strain was monitored using the Biolector microplate reader (M2P labs). Biomass was measured every 10 min for 24 hours at a wavelength of 620 nm, keeping the plate under continuous shaking. The following settings were used: 30 °C, 85 % humidity, 20.95 % O2 and 1,000 rpm.

**FluorescenceMicroscopy**

Single yeast colonies were grown overnight in 5 ml SD-Raf at 30°C under shaking at 180 rpm. The following day cells were transferred to SD-Gal to a final OD600 of 0.1. The yeast suspension was then introduced into a CellASIC Microfluidics Yeast Plate Y04C (Merck Millipore, Germany) and cells were monitored with a 60xoil immersion objective on an inverted epifluorescence microscope (Nikon Eclipse Ti) equipped with a Hamamatsu C11446 Orca-Flash 4.0 digital camera. Images were processed with the open source image processing package Fiji.

**GFP-pull down and sample preparation for mass spectrometry analysis**

The expression of αSynuclein, TDP43 and HTT fused to a GFP tag was induced by addition of galactose to the growth medium to a final concentration 2%. Cells were harvested after 3 and 6 h of overexpression induction. Protein extracts were prepared by glass-bead lysis of the collected cellular pellets in lysis buffer (150 mM HEPES pH 7.5, 150 mM NaCl, 0.4% NP40, 0.5 mM TCEP) plus 1× Complete, EDTA-free protease-inhibitor mix (Roche) and cleared by centrifugation at 2000 g for two minutes.

GFP fusion proteins and their interaction partners were immunopurified by addition of 15 μl slurry of GFP-Trap MA (Chromotek) and incubated for 2 h at 4 °C. This was followed by three wash steps in wash buffer (100 mM HEPES pH 7.5, 150 mM NaCl, 0.04% NP40, 0.5 mM TCEP (Tris(2-carboxyethyl) phosphine)) plus 1× Complete, EDTA-free protease-inhibitor mix (Roche).

The immune-affinity purified material was resuspended in 30 µl 0.1 M Ammonium bicarbonate pH 8.0 and incubated with 50 ng of LysC for 1 h at 32°C. For disulfide reduction 1 mM TCEP (Tris(2-carboxyethyl) phosphine) was added to the supernatant (eluted proteins) and incubated for 30 min at 37°C. Free sulfhydryl groups were alkylated with 10 mM Iodoacetamide for 30 min at room temperature in the dark. Initially, the proteins were digested with 30 ng LysC for 14 h at 25 °C followed by a second digestion with 25 ng trypsin for 5 hours at 25 °C.

After acidification with 2% formic acid, peptides were cleaned-up with C-18 ZipTips pipette tips (Millipore). Prior to peptide loading ZipTips were equilibrated with 80% acetonitrile and 0.1% formic acid. Bound peptides were washed with 0.1% formic acid and eluted with 80% acetonitrile - 0.1% formic acid. Samples were dried in a vacuum centrifuge, solubilized in 0.1% formic acid, and analysed by mass spectrometry.

**Heat Shock Experiment**

For the heat shock (HS) experiment the *S. cerevisiae* W303 strain was used. Cells were grown in SD-Glu over night at 30°C under shaking at 180 rpm. The following day cells were transferred to SD-Glu to a final OD600 of 0.4. When the culture reached OD600 0.9, cells were centrifuged at 3000 g for five min and resuspended in 10 ml SD-Glu medium prewarmed to 42°C. The yeast suspension was then introduced into one liter of SD-Glu medium prewarmed to 42°C and incubated at 42°C. As control, 10 ml of the exponentially grown culture was resuspended into one liter SD-Glu medium. After 0 min, 30 min and one hour, 500 ml of both the heat-shocked and the control cultures were harvested for further experiments (see the “Sample preparation for LC-MS/MS” session). The remaining cells were centrifuged at 3000 g for five min and transferred to SD-Glu medium prewarmed to 30°C and incubated at 30°C for two hours under shaking at 180 rpm to allow cells to recover from heat shock. Cells were then harvested for the proteomic sample preparation step.

**PROTEOMIC ANALYSIS**

**Sample Preparation for LC-MS/MS**

Cells were quenched by adding pure trichloracetic acid (Sigma Aldrich) to the yeast cultures to a final concentration of 10% (v/v) and incubating for 10 min on ice. Samples were then centrifuged at 3000 g for 5 min at 4°C and the supernatant was discarded. The pellet was washed twice with 10 ml cold acetone before being transferred into a new tube. After an additional centrifugation step at 3000 g for 5 min at 4°C the acetone was removed and the pellet was further processed for protein extraction.

**Cell Lysis and Protein Extraction**

To lyse the cells, the pellet was first mixed with acid-washed glass beads (Sigma Aldrich) and 500 μl of lysis buffer containing 8M urea, 50 mM ammonium bicarbonate and 5 mM EDTA. The mixture was then transferred to a FastPrep-24TM 5G Instrument (MP Biomedicals) where the cells were disrupted at 4°C by 6 rounds of beads-beating at 30 seconds with 120 seconds pause between the runs. The samples were then centrifuged for 5 min at 16'000 x g at 4°C to remove cell debris and the supernatant was transferred into a new tube. This process was repeated three times to increase the lysis efficiency and the overall protein amount. The protein concentration was determined with bicinchoninic acid Protein Assay Kit (Thermo Scientific) following the manufacturer’s protocol.

**In Solution Protein Digestion**

100 µg of protein extracts were subjected to digestion. Samples were vortexed and sonicated for 5 min. In the first step, dithiothreitol (Sigma Aldrich) was added to a final concentration of 12 mM and incubated for 30 min at 37 °C to reduce the disulfide bridges followed by the alkylation of free cysteine residues with iodoacetamide (Sigma Aldrich) at 40 mM final concentration (45 min at 25°C). The samples were then diluted 1:8 with freshly prepared 0.1 M ABC. Sequencing grade trypsin (Promega) was added at an enzyme to substrate (E/S) ratio of 1/100 and digested at 30°C overnight. The digestion was stopped by adding formic acid (FA, Sigma Aldrich) to a final concentration of 2%.

**Peptide Cleanup**

The digested samples were loaded onto SepPak C18 columns (Waters) that were previously washed twice with 100% acetonitrile (ACN, Sigma Aldrich) and equilibrated 3 times with a 0.2% FA solution. The peptides bound to C18 resins were washed 5 times with a 0.2% FA solution and eluted twice with 300 μl 60% ACN. The elution was dried down in a vacuum centrifuge (Thermo Scientific) and peptides were resuspended in a 0.15% FA solution to an approximate concentration of 1 mg/ml and transferred to MS vials.

**Phosphopeptide Enrichment**

After the peptide clean-up step, each sample was diluted in 280 μl phtalic acid (PA) solution (86.7 mg/ml PA, 80% ACN, 3.5% trifluoroacetic acid (TFA, Thermo Scientific)) by vortexing and sonicating for 5 min. The peptide solution was centrifuged at 16'000 x g for 5 min to remove solid debris. The peptide solution was transferred to Mobicol spin columns containing titaniumdioxide (TiO2) beads (GL Science) that had been washed with 280 μl of methanol by vortexing in short pulses and centrifuged at 800 g, and equilibrated with 280 μl of PA solution by vortexing in short pulses and centrifuged at 800 g. Transfer of peptide solution to the spin columns was achieved as follows: the bottom of the Mobicol columns was closed with a small plug and the columns were vortexed to mix the peptide solution with the TiO2 beads and incubated for 30 min at room temperature under end-over-end rotation. After incubation, the beads were washed twice with 280 μl of PA solution (load, vortex, spin down at 800 g) followed by two washing steps with 280 μl of 80% ACN, 0.1% TFA, two washing steps with 280 μl of 40% ACN, 0.1% TFA and two washing steps with 280 μl of 0.1% TFA. For the elution, 280 μl of 0.3 M NH4OH were added to the beads, which were then incubated for 3 min at room temperature and centrifuged at 800 g. The elution was performed a second time with the same parameters. The solution was acidified immediately after elution with 40 μl of 25% TFA.

**Phosphopeptide Cleanup**

The cleanup of phosphopeptides was performed as described in the “Peptide cleanup” session with UltraMicroSpin C18 columns (Waters) and using a 0.1% TFA solution for equilibration and washing, and a 50% ACN solution for the elution step.

**LC-MS/MS Measurements**

**Q Exactive Measurements**

The peptide and phosphopeptide samples were measured in a data-dependent acquisition shotgun mode on a Q Exactive (QE, Thermo Fisher Scientific) mass spectrometer equipped with a nano-electrospray ion source and coupled to a EASY-nLC 1000 (Thermo Fisher Scientific) nano-flow liquid chromatography system. Peptides were loaded into a 50 cm fused silica column with a 75 μm diameter (New Objective) filled with C18 beads (Michrom Bioresources) with a diameter of 1.9 μm and heated by a column heater to 40°C. The peptides were separated by a linear gradient of ACN/H2O with 0.1% FA, using a gradient from 3 to 35% ACN in 210 min total method duration (washing, equilibration) with a flow rate of 300 nl/min. The QE was operated in the full MS mode with a resolution of 70000 at a scan range on 400 to 2000 m/z with the maximum injection time (IT) of 250 ms and maximum AGC (automatic gain control) target of 1e6. In the MS/MS mode the QE was operated with a resolution of 17500, a maximum IT of 60 ms and AGC target of 5e4. The top 10 most abundant precursor ions with a charge ≥ 2 and an isolation window of 3 m/z were selected for fragmentation from the full MS scan. The dynamic exclusion time was set to 40 s to avoid redundant measurements of individual precursors.

For the GFP pull down experiments the following gradient of an acetonitrile/water mix was used for separation: linear gradient from 5 to 8% buffer B over 2 minutes, linear gradient from 8 to 25% buffer B over 68 minutes, linear gradient from 25 to 40% buffer B over 10 minutes, linear gradient from 40 to 90% buffer B over 5 minutes and isocratic for 5 minutes. Buffer A was 0.1% formic acid and buffer B was 0.1% formic acid in 100% acetonitrile. The flow rate was 300 nL/min and the column was heated to 50 °C. The mass spectrometer was operated in data-dependent acquisition mode.

MS1 spectra were acquired from 350 to 1500 m/z at a resolution of 70000. The 20 most intense precursors were selected for Collision-induced dissociation fragmentation and the corresponding MS2 spectra were acquired at a resolution of 17500 using maximally 100000 ions, collected for maximally 55 ms. All multiply charged ions were used to trigger MS-MS scans followed by a dynamic exclusion for 30 s. Singly charged precursor ions and ions of undefinable charged states were excluded from fragmentation.

**TripleTOF Measurements**

The peptide samples were measured in a data-dependent acquisition shotgun mode on a 5600 TripleTOF mass spectrometer (Ab Sciex) with a nano-electrospray ion source. The chromatographic peptide separation was performed in an Eksigent 1D-plus Nano liquid chromatography system (AB Sciex) equipped with a 15 cm fused-silica column with 75 μm inner diameter (BGB Analytic) packed in-house with 5 μm Magic C18 AQ beads (Michrom Bioresources). Approximately 3 μg of peptides were loaded from an AS-2 autosampler cooled to 4°C (ABSciex) and separated on the chromatography column with a linear gradient of ACN/H2O with 0.1% FA, using a gradient from 5 to 35% ACN in 120 min total method duration (washing, equilibration) with a flow rate of 300 nl/min. The accumulation time for TOF scans was set to 0.299995 s. The mass range was set to 400-1250 Da. Peptides with a charge of 2-5 and a signal exceeding 150 cps were selected for fragmentation. Up to 20 precursor ions were monitored and excluded for 20 s after detection. The fragment ion scan was performed with an accumulation time of 0.149998 s and a mass range of 170-1500 Da in a high sensitive mode with a total cycle time of 3.35 s.

**Data Analysis**

For label-free data quantification the raw files from the MS measurements (TTOF: .wiff; QE: .raw) were imported into Progenesis LC-MS software (Nonlinear Dynamics, Waters, Durham, USA). The software is capable of aligning the MS measurements, detecting the precursors and extracting the intensity of the individual peptides. For the peak picking parameters, the peak picking limits were set to “default” and the maximum precursor charge was set to 5. For peptide identification and quantitation, MS2 data were exported for database search with Mascot (Matrix Science) in the .mgf format. The parameters for the .mgf files were set as follows: the count of fragment ions was limited to 200, the peptide rank was chosen to be less than 3 and the charge state was set to 2 - 5. This filtering resulted in smaller .mgf files with high confidence spectra and without any impact on the search results. The parameters for the database search in Mascot were the following: the database was the full yeast proteome (SGD database, downloaded 14.12.2011, 6440 protein sequences) containing reverse decoy sequences for each protein. The protease used for digestion was trypsin set to fully tryptic cleavages. Up to two missed cleavages were allowed. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines as a variable modification. In case of phosphopeptide search, the phosphorylation of serines, threonines and tyrosines was added as a variable modification. The peptide tolerance was set to 30 ppm for MS1 and 0.8 Da for MS2 in case of measurements on the TTOF and 10 ppm for MS1 and 0.02 Da for MS2 for measurements on the QE. The peptide charge included 2+, 3+ and 4+ ions and the monoisotopic mass was used. The data format was Mascot generic and the instrument type was set to ESI-QUAD-TOF for the TTOF and ESI-FTICR for the QE. The search results were exported in the .xml format using an ion score cut-off of 10 and re-imported into the Progenesis environment resulting in peptide identifications mapped to the detected features. Before summing up all peptide intensities for protein quantitation, the false discovery rate (FDR) was adjusted to 1% based on the decoy database by reducing the mass error and the ion score until 1% FDR was reached. The final protein and phosphopeptide tables were exported in the .csv format for post-processing with an R-framework based analysis tool SafeQuant https://github.com/eahrne/SafeQuant/. SafeQuant was used for statistical validation of differentially expressed proteins and phosphopeptides across the individual conditions. An expression ratio was calculated based on the median intensity of the biological triplicates and subjected to statistical validation taking into the account the variation of peptide intensities per condition and total number of detected features. Each calculated expression level was associated with a significance q-value (p-value adjusted for multiple testing) based on a modified t-test using the empirical Bayes method and multiple testing using the Benjamin-Hochberg method (Benjamini and Hochberg, 1995). The output of this statistical analysis was filtered for significant protein and phosphopeptide changes (q-value < 0.05) and fold change cut-off of two.

For the GFP-pull down experiments the collected DDA spectra were searched against the *S.cerevisiae* S288C reference proteome Uniprot FASTA database (Version: November 2015) and a list of common protein contaminants (exported from the MaxQuant software proteomics package) using the Sorcerer-SEQUEST database search engine (Thermo Electron). Trypsin was set as the digesting protease with a tolerance of two missed cleavages and not allowing for cleavages of KP and RP peptide bonds. The monoisotopic peptide and fragment mass tolerances were set to 10 p.p.m. and 0.02 Da, respectively. Carbamidomethylation of cysteines (+57.021 Da) was defined as a fixed modification and the oxidation of methionines (+15.995) as a variable modification. Protein identifications were statistically analysed with Percolator and filtered to a cutoff of a false discovery rate of <1% calculated based on a target-decoy approach. The number of peptides observed in each pull-down, counted as spectral counts, were integrated to quantify the amount of protein present. The protein quantification experiment relative to each bait was normalized to the number of spectral counts detected for the same protein in an experiment performed in the same environmental condition using a strain where the protein was not GFP tagged.

BIOINFORMATIC ANALYSIS

The visualization, exploration and statistical analysis of the data produced after the significance analysis of protein and phosphopeptide abundances were performed using R (version 3.3.1), Python (version 2.7) and the Python library Pandas (version 0.18.1). The hierarchical clustering analysis of both protein abundance variations and GO enrichments was performed using the open source python-based SciPy library and visualized with the Seaborn graphic library.

**Analysis of the kinase-substrate network**

The NetworKIN algorithm was applied to the phosphoproteomics dataset to infer kinase-substrate relationships. A high quality interaction network (interactome) was constructed as proposed by Stuart and collaborators (Stuart et al., 2009) using curated interaction data available in both SGD (Saccharomyces Genome Database) and YEASTRACT (www.yeastract.com) websites. The interaction\_data.tab file, containing the interaction data incorporated from BioGRID, was downloaded from SGD. The list of transcription factors-target interactions (TF interactions) was downloaded from the yeast transcription factors YEASTRACT database (RegulationTwoColumnTable\_Documented\_2013927.tsv.gz). The interactome was imported in Cytoscape 3.2.0 (Shannon et al., 2003) to perform network analysis of all the predicted kinases and the signficant p-sites.

**Gene Ontology Enrichment Analysis**

The gene ontology (GO) enrichment analysis was performed using the GOATOOLS python-based library (Klopfenstein et al., 2018). The background set correspond to all yeast proteins. The p-value was calculated using Fisher's exact test and then adjusted for multiple testing using the Benjamin-Hochberg correction method (Benjamini and Hochberg, 1995). To compare and visualize the results of the functional enrichment analysis the list of significantly enriched GO terms (adjusted p-value < 0.05) for every condition was converted to a vector reporting the -log10(adjusted p-value) of every term. GO terms with a number of associated genes bigger than 500 were excluded from the analysis to reduce the complexity and the redundancy of the result but preserving the biological outcome. The Euclidean distance between all the vectors was computed and the hierarchical clustering of the GO terms enriched in the different models was performed using the ‘average’ linkage method from the open source python-based SciPy library. The result of the clustering analysis was visualized with the “clustermap” function of the Seaborn package.