Supplemental Figure Legends

Figure S1. Long-lived eye lens crystallins. Related to Figure 1. (A) Schematic of the location of the eye lens within the eye organ. (B) Crystallin purification. The soluble protein fraction from rat eye lenses were analyzed by SDS-PAGE and stained with coomassie, showing the relative simplicity of eye lens proteome. (C) Eye lens crystallins are long-lived. ^{15}N fractional abundance was determined for all crystallins from eyes of a 6-months post-chase animal. Error bars represent standard deviations between peptides. (D) Crystallin levels through the chase. MS1 elution profile traces from an example peptide (R.PNYQGHQYFLR.R) of crystallin-gamma-B are plotted at 0, 6, and 12 months post-chase. ^{15}N signal is plotted in orange and ^{14}N signal in grey.

Figure S2. The scaffold NPC components at 6 months. Related to Figure 1. (A) Peripheral versus scaffold Nups. Example MS1 elution profile traces plotted as previously described for a non-long-lived peripheral Nup (left, Pom121), and a long-lived scaffold Nup (right, Nup205) from a 6-months post-chase rat. (B) Long-lived Nups. Schematic of the NPC with the Nup205 complex highlighted in dark orange and the Nup107/160 complex in light orange. Relative ^{15}N fractional abundances of members of these complexes at 6-months post-chase are listed on the left, determined as an average of ^{15}N fractional abundance of peptides across 1-3 animals and non-sorted nuclei when possible. (C) The Nup205 complex and the (D) Nup107/160 complex. Representative MS1 elution profile traces are plotted as previously described for 3 other members of the Nup205 complex (C), or 4 other members of the Nup107/160 complex (D) from 6-months post-chase rats.

Figure S3. Other long-lived proteins. Related to Figure 1. (A-D) MS1 elution profiles are plotted as described earlier for, (A) Lamin proteins B1 and B2, (B) myelin proteins myelin oligodendrocyte glycoprotein and myelin basic protein, and (C) enzyme Cnp1.

Figure S4. Longevity differences in neurons versus glia. Related to Figure 4. (A) Confirmation of NeuN sorting. Brain nuclei were purified from a 1-year post-chase rat, labeled with NeuN-488, and FACS sorted into high (NeuN positive) and low (NeuN negative) populations. The sort was confirmed by staining with the DNA dye Hoechst and imaging sorted and fixed nuclei by confocal microscopy.

Left: NeuN positive sorted nuclei. Right: NeuN negative sorted nuclei. (B-C) Variability of ^{15}N fractional abundance over multiple animals. MS was performed on sorted brain nuclei from 3 different rats at 1-year post-chase and the indicated Nups quantitated for ^{15}N fractional abundance by averaging data for multiple peptides within each animal from NeuN positive (B), and negative (C) nuclei. Error bars represent standard deviations. (D) H3.1 identification. Example histone H3.1 MS2 fragmentation spectrum is plotted, showing y (red) and b (blue) ions that match the only H3.1-unique peptide (depicted).

Figure S5. NPC counting throughout age. Related to Figure 6. NPCs were counted, per nucleus, of liver and brain nuclei from rats of 4 weeks, 6, 13, and 24 months of age. Plotted are the average surface areas of the quantitated nuclei (A) and average total pore numbers per nucleus (B). Error bars represent standard deviations.

Extended Experimental Procedures

Pulse-chase labeling of rats: Rats were pulse labeled as described earlier (McClatchy et al., 2007; Savas et al., 2012), and switched to a normal ^{14}N diet (chase) at 6 weeks post-natal. Rats from multiple litters were then sacrificed at 4, 6, 9, and 12 months post-chase, tissues harvested, and flash frozen.

Tissue fractionations and purifications: Eye lens crystallins were isolated from intact eyes by first dissecting away the lens from the reminder of the eye. The lens was then homogenized in a 1.5mL centrifuge tube with a handheld plastic homogenizer, and centrifuged at 20,000rcf for 5 minutes. The supernatant represented the soluble crystallin fraction. Liver and brain tissues were fractionated by thawing on ice and homogenizing with a glass dounce homogenizer in nuclei purification buffer. Liver and brain nuclei were purified according to protocol (Blobel and Potter, 1966; Lovtrup-Rein and McEwen, 1966). Supernatants of the nuclei purifications were diluted 5x with corresponding nuclei purification buffer with no sucrose, and spun at 13,000rcf for 15 minutes. The pellet was resuspended in nuclei purification buffer, representing the mitochondria-enriched fraction. The supernatant was then spun at 100,000rcf for 20 minutes, and the pellet resuspended in nuclei purification buffer representing the ER-enriched fraction. The supernatant of this fraction represented the cytosol-enriched fraction. For chromatin fractions, nuclei were digested with DNase I for 10 minutes at room temperature, and then spun down at 20,000rcf for 5 minutes. The supernatant was considered the chromatin fraction. Histone fractions were isolated through salt extraction as described elsewhere.

NeuN nuclei labeling, sorting, and analysis: Freshly isolated brain nuclei were spun down at 800rcf for 5 minutes, and resuspended in TKM0.25 buffer (50mM Tris pH 7.5, 25mM KCl, 1mM $MgCl_2$, 250mM sucrose) with Alexa Fluor488-conjugated NeuN antibody (Millipore) diluted 1000x, and incubated in the dark without disturbing for 1 hour. Nuclei were then sorted as described elsewhere using a Vantage SE DiVa using TKM0.25 as sheath fluid (Spalding et al., 2005). Collected nuclei were spun down at 800rcf for 10 minutes, and the pellet resuspended in 100uL TKM0.25, and a small amount quantitated for protein content and fixed for microscopy. Fixed nuclei from NeuN positive and

negative sorts were incubated with Hoechst dye, spotted on slides, and immediately sealed with coverslips. Coverslips were imaged on a Zeiss LSM 710 laser-scanning confocal microscope.

Mass spectrometry: All MS samples were digested and analyzed as described previously (Savas et al., 2012). Solid urea (8 M) was added to samples for LCLC-MS/MS analysis, and extracts were processed with ProteasMAX (Promega, Madison, WI, USA) per the manufacturer's instruction. The samples were subsequently reduced by TCEP (tris(2 carboxyethyl)phosphine, 5 mM, room temperature, 20 min), alkylated in the dark by 10mM iodoacetamide (10 mM, 20 min), digested with Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) overnight at 37 °C, and the reaction was stopped by acidification to 5% final.

MudPIT and LTQ Velos Orbitrap MS

The protein digest was pressure-loaded into a 250-µm i.d capillary packed with 2.5 cm of 10-µm Jupiter C18 resin (Phenomenex, Torrance, CA, USA) followed by an additional 2.5 cm of 5-µm Partisphere strong cation exchanger (Whatman, Clifton, NJ). The column was washed with buffer containing 95% water, 5% acetonitrile, and 0.1% formic acid. After washing, a 100-µm i.d capillary with a 5-µm pulled tip packed with 15 cm of 4-µm Jupiter C18 resin (Phenomenex, Torrance, CA, USA) was attached to the filter union and the entire split-column (desalting column–union–analytical column) was placed inline with an Agilent 1200 quaternary HPLC (Palo Alto, CA) and analyzed using a modified 11-step separation described previously (Link et al., 1999; Washburn et al., 2001). The buffer solutions used were 5% acetonitrile/0.1% formic acid (buffer A), 80% acetonitrile/0.1% formic acid (buffer B), and 500 mM ammonium acetate/5% acetonitrile/0.1% formic acid (buffer C). Step 1 consisted of a 90 min gradient from 0-100% buffer B. Steps 2-11 had a similar profile with the following changes: 5 min in 100% buffer A, 3 min in X% buffer C, a 10-min gradient from 0-15% buffer B, and a 108-min gradient from 15-100% buffer B. The 3-min buffer C percentages (X) were 10, 20, 30, 40, 50, 60, 70, 80, 90, 100%, respectively for the 11-step analysis. As peptides eluted from the microcapillary column, they were electrosprayed directly into an LTQ Velos Orbitrap mass spectrometer (Thermo Finnigan, Palo Alto, CA) with the application of a distal 2.4-kV spray voltage. A

cycle of one full-scan mass spectrum (400-1800 m/z) at a resolution of 60,000 followed by 15 data dependent MS/MS spectra at a 35% normalized collision energy was repeated continuously throughout each step of the multidimensional separation. Maximum ion accumulation times were set to 500 ms for survey MS scans and to 100 ms for MS2 scans. Charge state rejection was set to omit singly charged ion species and ions for which a charge state could not be determined for MS/MS. Minimal signal for fragmentation was set to 1,000. Dynamic exclusion was enabled with a repeat count: 1, duration: 20.00S, list size: 300, exclusion duration 30.00S, exclusion mass with high/low: 1.5m/z. Application of mass spectrometer scan functions and HPLC solvent gradients were controlled by the Xcaliber data system.

Analysis of tandem mass spectra

Protein identification and quantification and analysis were done with Integrated Proteomics Pipeline - IP2 (Integrated Proteomics Applications, Inc., San Diego, CA. http://www.integratedproteomics.com/) using ProLuCID, DTASelect2, Census, and QuantCompare. Spectrum raw files were extracted into ms1 and ms2 files from raw files using RawExtract 1.9.9 (http://fields.scripps.edu/downloads.php) (McDonald et al., 2004), and the tandem mass spectra were searched against EBI IPI mouse protein database (EBI-IPI_rat_3.30_con_06-28-2007). In order to accurately estimate peptide probabilities and false discovery rates, we used a target/decoy database containing the reversed sequences of all the proteins appended to the target database (Peng et al., 2003). Tandem mass spectra were matched to sequences using the ProLuCID (Xu et al., 2006) algorithm with 50 ppm peptide mass tolerance for precursor ions and 400 ppm for fragment ions.

ProLuCID searches were done on an Intel Xeon cluster running under the Linux operating system. The search space included all fully- and half-tryptic peptide candidates that fell within the mass tolerance window with no miscleavage constraint. Carbamidomethylation (+57.02146 Da) of cysteine was considered as a static modification.

The validity of peptide/spectrum matches (PSMs) was assessed in DTASelect (Cociorva et al., 2007; Tabb et al., 2002) using two SEQUEST (Eng et al., 1994) defined parameters, the cross-

correlation score (XCorr), and normalized difference in cross-correlation scores (DeltaCN). The search results were grouped by charge state (+1, +2, +3, and greater than +3) and tryptic status (fully tryptic, half-tryptic, and non-tryptic), resulting in 12 distinct sub-groups. In each one of these sub-groups, the distribution of Xcorr, DeltaCN, and DeltaMass values for (a) direct and (b) decoy database PSMs was obtained, then the direct and decoy subsets were separated by discriminant analysis. Full separation of the direct and decoy PSM subsets is not generally possible; therefore, peptide match probabilities were calculated based on a nonparametric fit of the direct and decoy score distributions. A peptide confidence of 0.95 was set as the minimum threshold. The false discovery rate was calculated as the percentage of reverse decoy PSMs among all the PSMs that passed the confidence threshold. Each protein identified was required to have a minimum of one peptide. After this last filtering step, we estimate that both the protein false discovery rates were below 1% for each sample analysis.

Each dataset was searched twice once against light and secondly against heavy protein databases. After filtering the results from SEQUEST using DTASelect2, ion chromatograms were generated using an updated version of a program previously written in our lab (MacCoss et al., 2003). This software, called Census (Park et al., 2006), is available from the authors for individual use and evaluation through an Institutional Software Transfer Agreement (see http://fields.scripps.edu/census for details).

First, the elemental compositions and corresponding isotopic distributions for both the unlabeled and labeled peptides were calculated and this information was then used to determine the appropriate m/z range from which to extract ion intensities, which included all isotopes with greater than 5% of the calculated isotope cluster base peak abundance. MS1 files were used to generate chromatograms from the m/z range surrounding both the unlabeled and labeled precursor peptides.

Census calculates peptide ion intensity ratios for each pair of extracted ion chromatograms. The heart of the program is a linear least squares correlation that is used to calculate the ratio (i.e., slope of the line) and closeness of fit (i.e., correlation coefficient (r)) between the data points of the

unlabeled and labeled ion chromatograms. Census allows users to filter peptide ratio measurements based on a correlation threshold; the correlation coefficient (values between zero and one) represent the quality of the correlation between the unlabeled and labeled chromatograms, and can be used to filter out poor quality measurements

Long-lived protein identification

Long-lived proteins were identified in brain fractions (nuclei, cytoplasm, ER, mitochondria, chromatin, histones, glia-enriched nuclei, and neuronal-enriched nuclei) from rats 6-months post-chase. Average peptide enrichments (APE) were calculated for all datasets, and a python script was written to filter the data as such. First, peptides with profile scores <0.8 were thrown out. Next, each peptide was deemed to have ^{15}N content if APE values were above 0.8 and calculated fractional abundance was greater than 5% ^{15}N. If such a peptide indicated ^{15}N content of 5-10% or 90-100%, it was classified as a “heavy” peptide. If the peptide indicated ^{15}N content of 10-90% it was only classified as heavy if the regression score was >0.8, otherwise it was thrown out. All peptides with profile scores >0.8, but with <5% ^{15}N were considered to be “light”. Proteins were only then flagged for being long-lived if they had more than 2 “heavy” peptides, more than 65% of the peptides for that protein were “heavy”, and the average ^{15}N for all peptides from that protein was >5%. This list of proteins was then visually analyzed to ensure that ^{15}N signal was more significant than noise. Proteins in which most peptides indicated almost no ^{15}N, but had one single highly ^{15}N-enriched peptide were also discarded as probable misassignments. Peptides quantified can be found in the accompanying database.

^{15}N fractional abundance quantifications

MS samples were run on single eye lenses from 0, 6, and 12-months post chase animals, and on 2-3 different animals each from 0, 4, 6, 9, and 12-months post chase animals, brain nuclei purified and sorted with NeuN as described above. MS was performed and analyzed by CENSUS as described above. For quantitation, data for all peptides from the indicated proteins were extracted for all time points and datasets unfiltered. Peptides were then filtered for only those with regression scores above

0.8, and corresponding area ratios converted to ^{15}N fractional abundance through the formula: FA=100*(1/(1+AR)) where FA=fractional abundance and AR=area ratio. Fractional abundances were averaged across all corresponding fractions for the same time point and standard deviations calculated. Peptides quantified can be found in the accompanying database.

Determination of translation rates: Liver and brain tissues were harvested from two 6 and 24-month old spraugh dwaly rats each, and immediately flash frozen. For ribosome footprinting, tissues were thawed on ice and homogenized in TKM0.25 with protease inhibitors (complete EDTA-free, Roche) and murine RNase inhibitors (NEB). RNase I (1000 units per 400 µL, Ambion) and Turbo DNase (9.6 units into 400µL, Ambion) were then added and the homogenate was incubated at room temperature for 45 minutes for footprinting. RNase inhibitors (Superase-in, Life Technologies) were then added, Triton X-100 added to 1%, and the mixture overlayed on top of a TKM1 sucrose cushion (TKM0.25 with 1M sucrose), and spun at 190,000rcf (ave) for 4 hours. RNA from the pellets was purified using the miRNeasy kit (Qiagen) and deep sequencing performed and analyzed as described elsewhere (Ingolia et al., 2009).

NPC counting: Nuclei were purified from liver and brain tissue as described earlier from at least 2 different rats of 6 weeks, 6, 13, or 24 months of age. Nuclei were fixed in 4% PFA/1xPBS for 1 minute, and then spun (1000rpm, 10minutes) onto low tolerance coverslips (Zeiss) treated with poly-L-lysine, underlayed with TKM0.25. Nuclei on coverslips were fixed again with 4% PFA for 1 minute at RT and permeabilized with IF buffer (1x PBS, 1% triton X-100, 0.2% SDS) for 20 minutes at RT. NPCs were stained with the antibody mab414 for 1 hour in IF buffer, washed, and secondary stained for an additional 1 hour. Coverslips were mounted with prolong gold (Invitrogen) and allowed to cure for 24 hours at RT. Nuclei were then imaged with a Zeiss Elyra structured illumination super resolution microscope. After 3D reconstructions, pore numbers were quantitated using the spot finder tool in Imaris (Bitplane) and surfaces area using the surfaces tool. At least 30 nuclei were quantitated from at least 2 rats for each time point.

NPC protein levels with age: Liver and brain nuclei were purified as described previously from 6 and 24 month-old rat tissues, and NPCs subsequently purified according the protocol (Cronshaw et al., 2002). Multiple lanes at multiple dilutions of all NPC samples were separated by SDS-PAGE and transferred to nitrocellulose membranes for analysis by western blot. Antibodies to indicated proteins were visualized by the Odyssey scanner (Li-cor), and bands quantitated using ImageJ. Band intensities for indicated proteins were normalized to Nup107 band intensity, all background subtracted.

NPC accessibility assay: Liver nuclei were purified as described previously. All subsequent buffers contained 1x protease and phosphatase inhibitors (Complete and phosSTOP, Roche) and 500nM tricostatin A. Ten million nuclei were then spun at 800rcf for 5 minutes at 4C, and the pellet resuspended in 100 uL TKM0.25. 20uL were set aside (no TCEP) while TCEP was added to the remaining to 10mM, and aliquoted into 20uL reactions. All tubes were incubated at RT for 10 minutes, and indicated amounts of PEG1k-maleimide (NANOCS) added to initiate crosslinking. Crosslinking was allowed to proceed at RT for 30 minutes, and was quenched by adding 2-mercaptoethanol to 50mM. Crosslinked nuclei were then spun at 800rcf for 5 minutes, and resuspended in DNase buffer (10mM Tris pH 7.5, 2.5mM $MgCl_2$, 0.5mM $CaCl_2$) and incubated at RT for 10 minutes. Nuclei were then spun down at 800rcf for 5 minutes. The pellet was resuspended in Heparin buffer (20mM triethanolamine pH 7.5, 0.1mM $MgCl_2$, 1mM DTT, 0.3mg/mL heparin, 10% sucrose) and spun down at 1000rcf for 5 minutes. The pellet was then resuspended in Triton buffer (20mM triethanolamine pH 7.5, 0.1mM $MgCl_2$, 1mM DTT, 3% triton X-100, 0.075% SDS, 10% sucrose) and spun down at 2000rcf for 5 minutes. The pellet was then resuspended in 20uL empigen buffer (20mM triethanolamine pH 7.5, 0.1mM $MgCl_2$, 1mM DTT, 0.3% empigen BB, 10% sucrose) and incubated on ice for 10 minutes. The mixture was then spun at 20,000rcf for 15 minutes, and the supernatant containing NPCs saved. NPCs were then analyzed by SDS-PAGE/western blot using primary antibodies indicated, infrared secondary antibodies (Rockland), and imaged using the Odyssey scanner (Li-cor).

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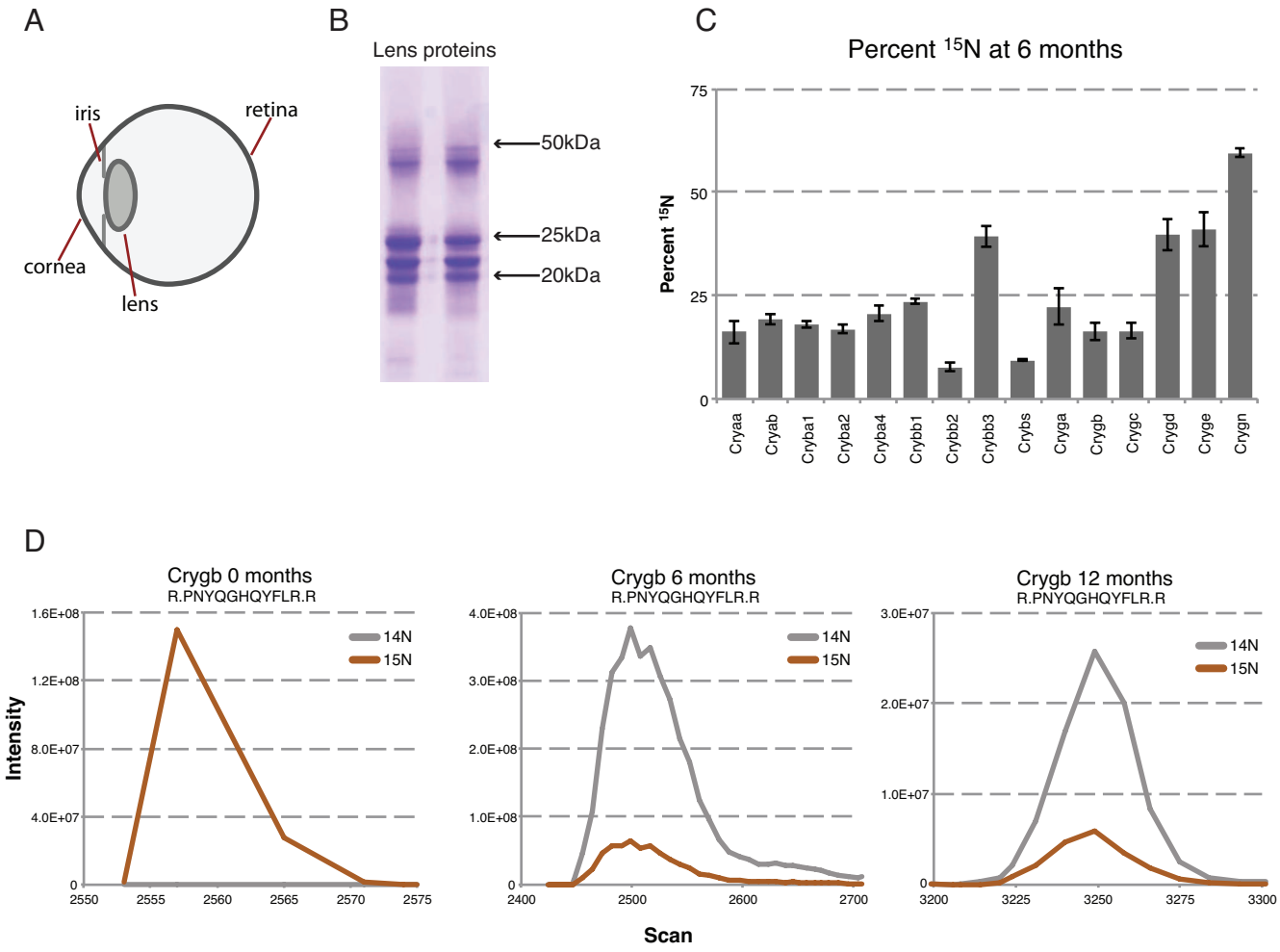
Figure S1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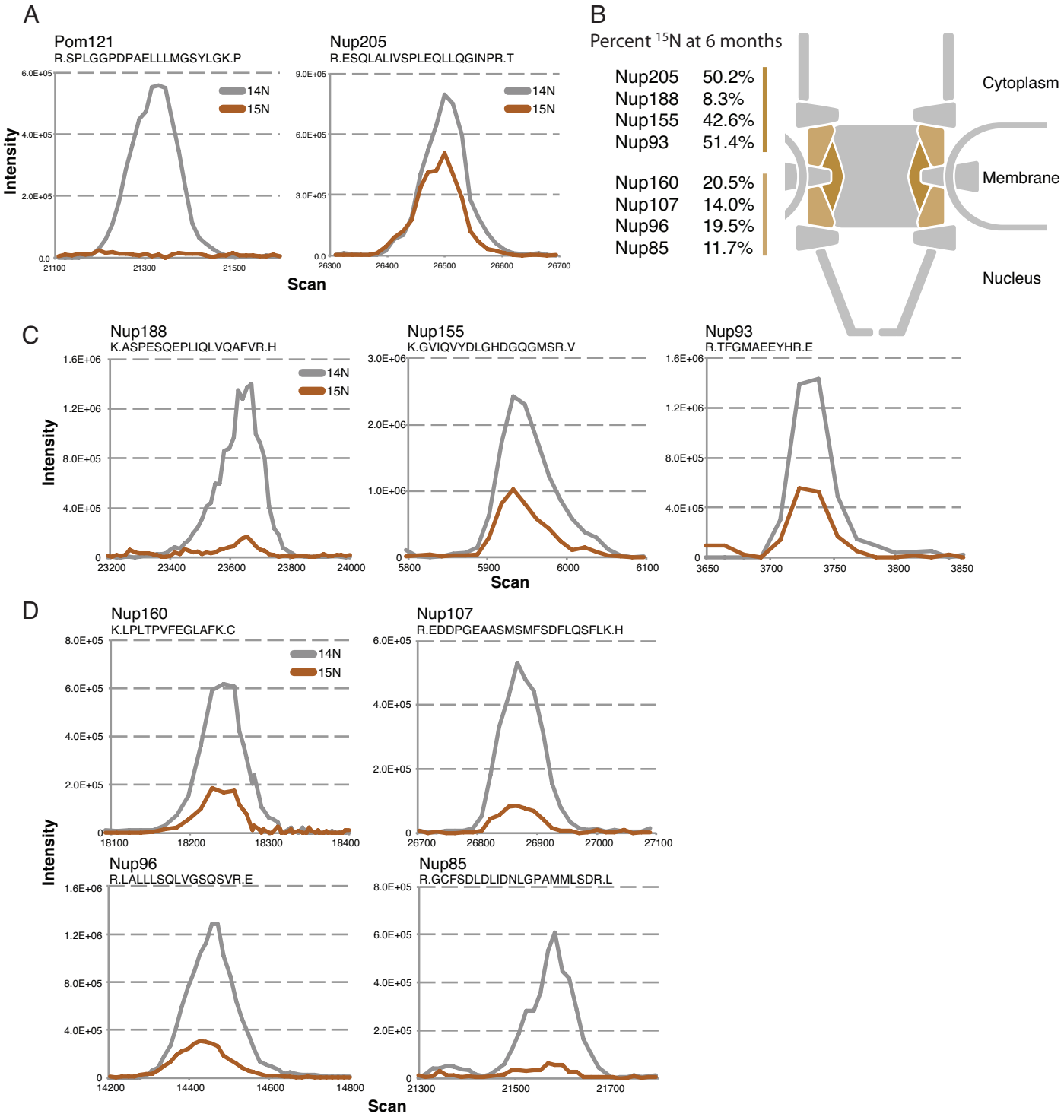
Supplemental Figure 2

Figure S2



Supplemental Figure 3

Figure S3

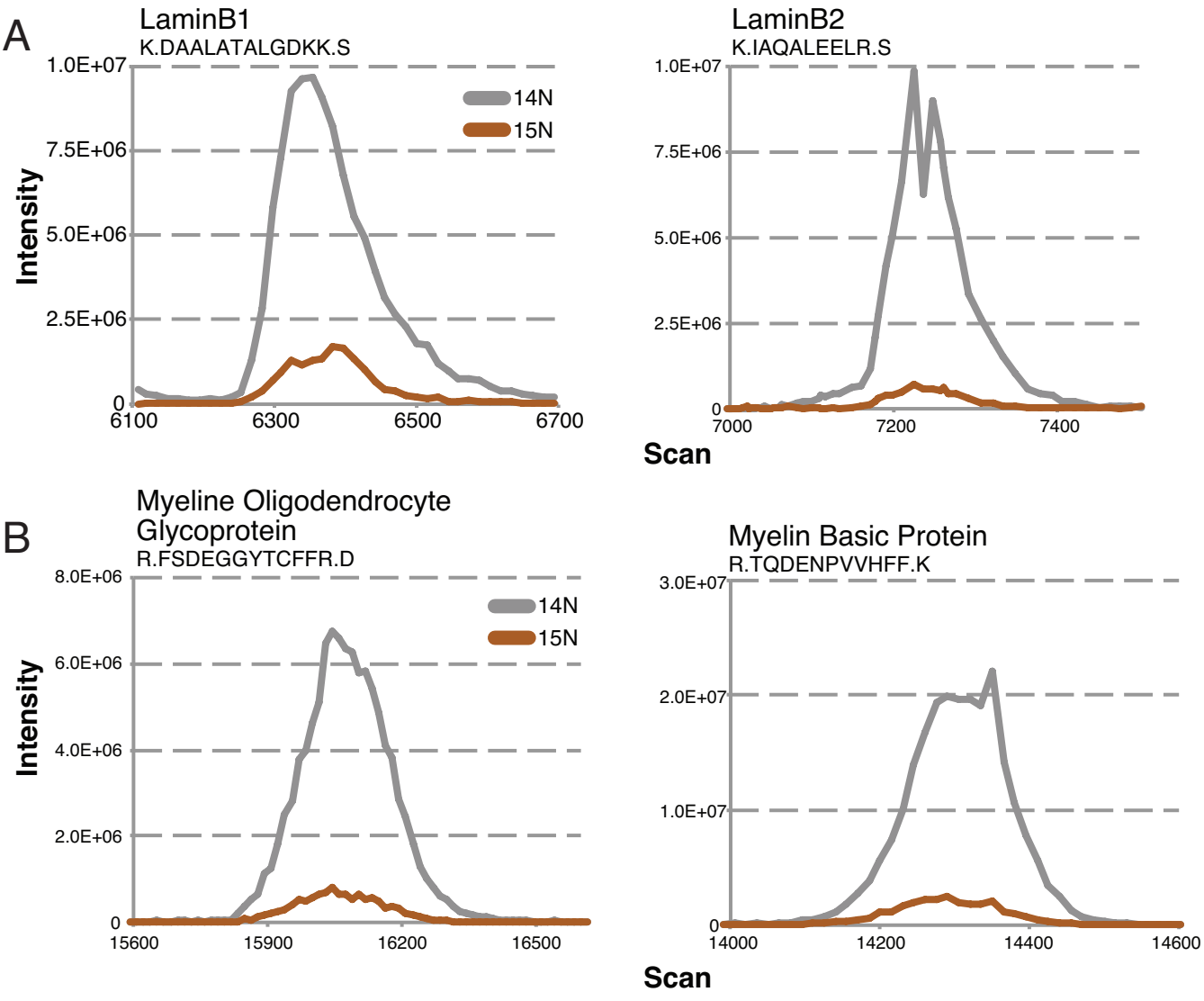
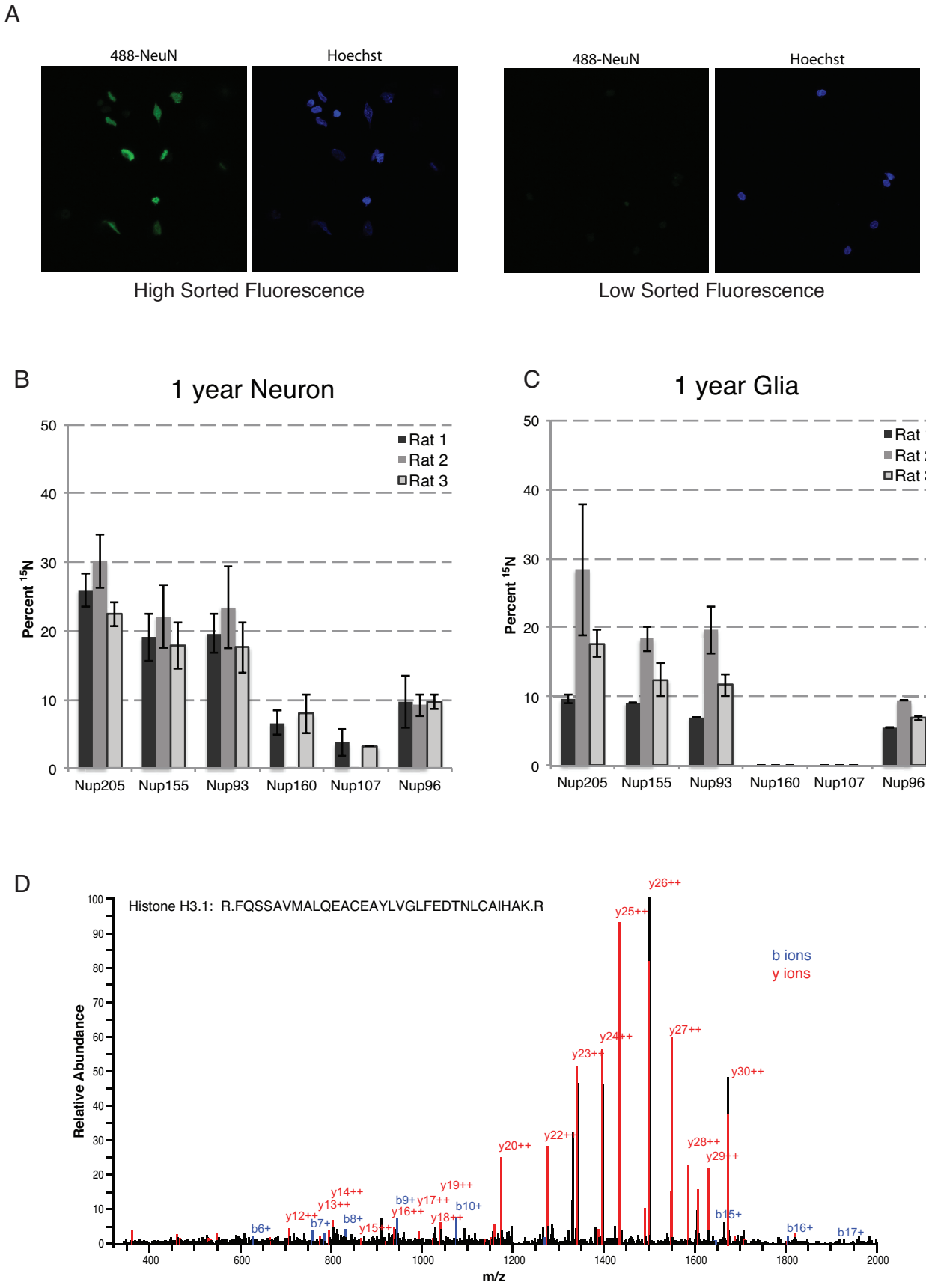


Figure S4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Relative Abundance
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Supplemental Figure 5

Figure S5

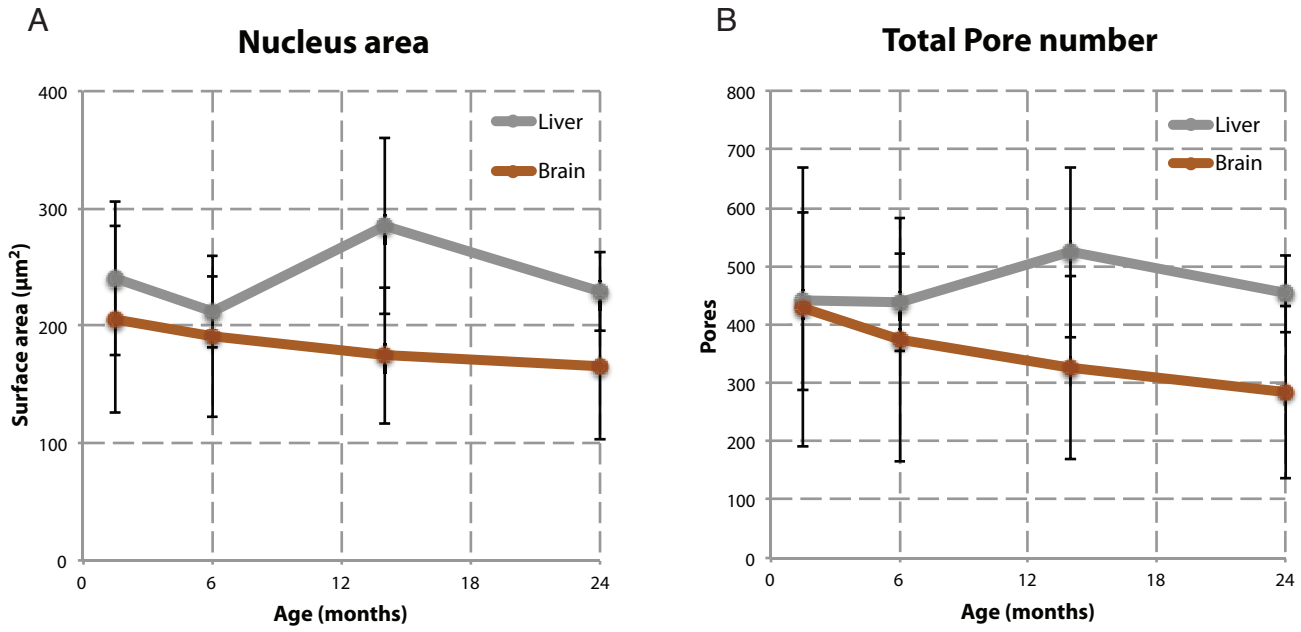
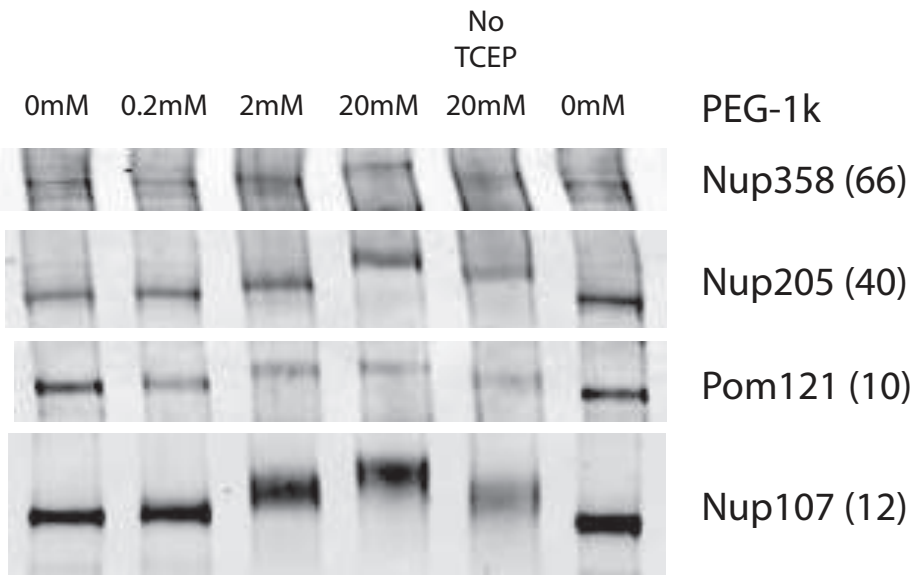


Figure S6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