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Supplemental Information

Noise Exposures Causing Hearing Loss Generate Proteotoxic Stress and Activate the Proteostasis Network

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Inventory of Supplemental Information

Supplemental Data: 7 Supplemental Figures and Legends

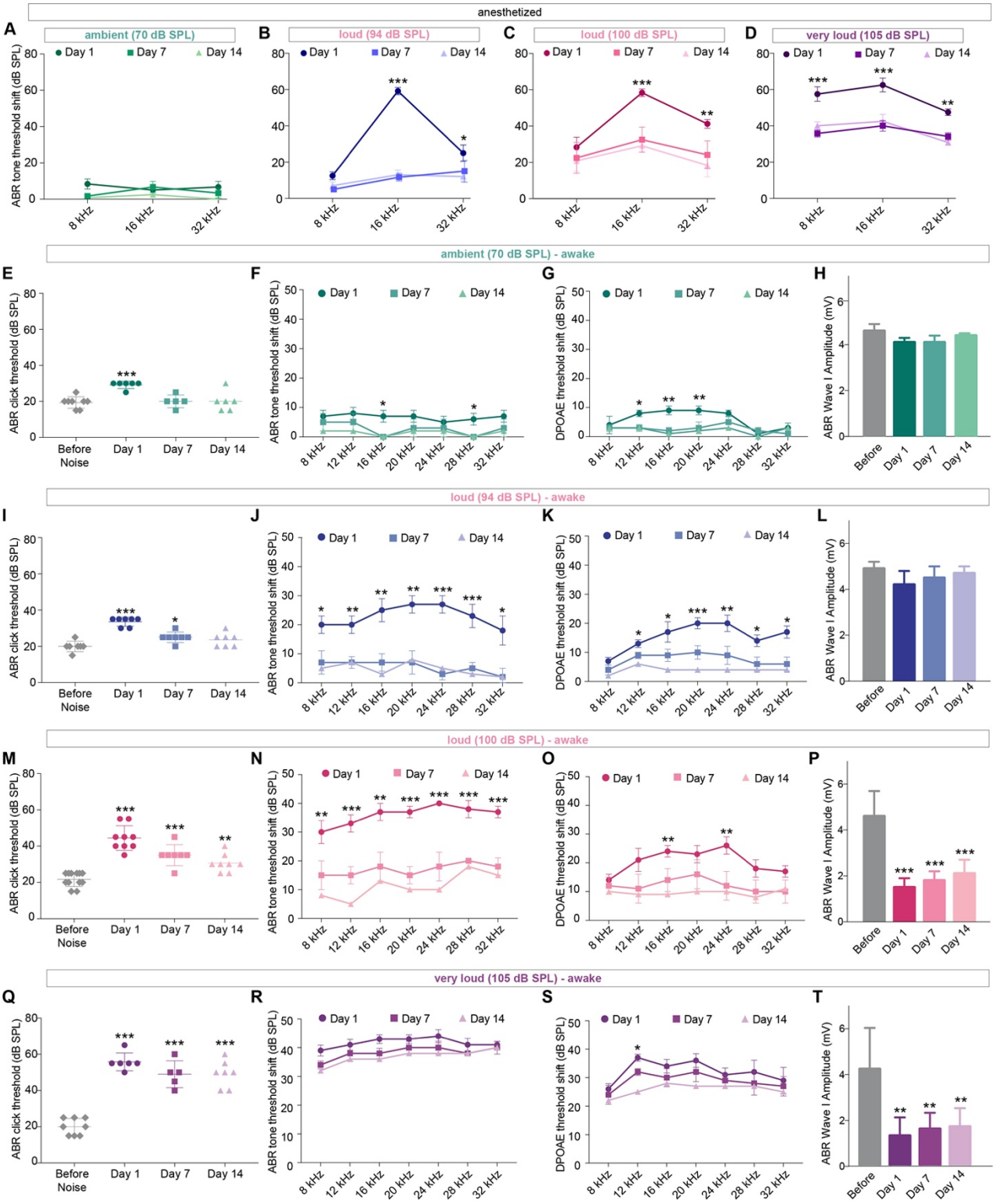

Figure S1 (related to Figure 1 – 7): ABR and DPOAE cochlear functional assays to determine responses in FVB mice after 30 minutes of exposure to ambient, loud, or very loud noise (8-16 kHz). (**A**) ABR tone recordings of anesthetized mice exposed to 70 dB SPL, (**B**) 94 dB SPL, (**C**) 100 dB SPL, and (**D**) 105 dB SPL (0.0632 1.00, 2.00, 3.55 Pa SIL respectively) white band noise centered on the 8-16 kHz range for 30 minutes. ABR tone threshold levels were significantly elevated at one compared to seven (A) and one compared to fourteen (B) days post exposure. (**E**) ABR click (**F**) ABR tone

(**G**) DPOAE and (**H**) Wave I amplitude of ABR click at 80 dB SPL stimulus after indicated recovery periods after noise exposure at 70 dB SPL level. The measurements show minimal shifts from baseline recordings most apparently one day after noise exposure with a return to baseline at day 7. (**I**) After 94 dB SPL noise exposure, level of ABR click threshold was significantly higher than before noise exposure. However, threshold shifts fully recovered to baseline levels by day 7. (**J**) ABR tone and (**K**) DPOAE threshold shifts had similar recovery patterns to ABR click. (**L**) ABR click wave I amplitude is not significantly reduced. (**M**) The 100 dB SPL condition provides a higher intensity of noise exposure and 20-40 dB shift in threshold levels by ABR click 1 day after noise exposure. Threshold shifts by ABR tone (**N**) and DPOAE (**O**) showed dramatic recovery 7 days after noise with incremental improvements between day 7 and 14. After 14 days of recovery, the lower frequency ranges almost completely recovered. However, the higher frequency range was more vulnerable to noise induced damage and recovery was not as robust. (**P**) ABR Click wave I amplitude showed a significant decrease with a gradual recovery after noise. At 105 dB SPL, our highest level of noise exposure, there was a dramatic increase in the threshold level of up to 50 dB in (**Q**) ABR click. (**R**) ABR tone and (**S**) DPOAE also revealed a strong threshold shift with a limited recovery capacity even 14 days after noise exposure, especially in the high frequency range. (**T**) Wave I amplitude was significantly reduced more than 50% compared to before noise exposure, with limited recovery that failed to reach the before noise levels. * = p value < 0.05, ** = p value < 0.01, *** = p value < 0.001, by one-way ANOVA with Bonferroni post hoc test was used to test for significant differences between before noise and after noise exposure conditions in ABR Click and measures of Wave I amplitude. One-way ANOVA was used to test for significant differences among after noise exposure conditions (day 1, 7 and 14) in ABR tone threshold and DPOAE. All data are presented as mean ± SE. N = 4 mice for (A), 5 - 6 mice for (B), 6 for (C-D), 5 - 8 for (E-H), 7 for (I-L), 7 - 11 for (M-P), and 5 - 8 for (Q-T).

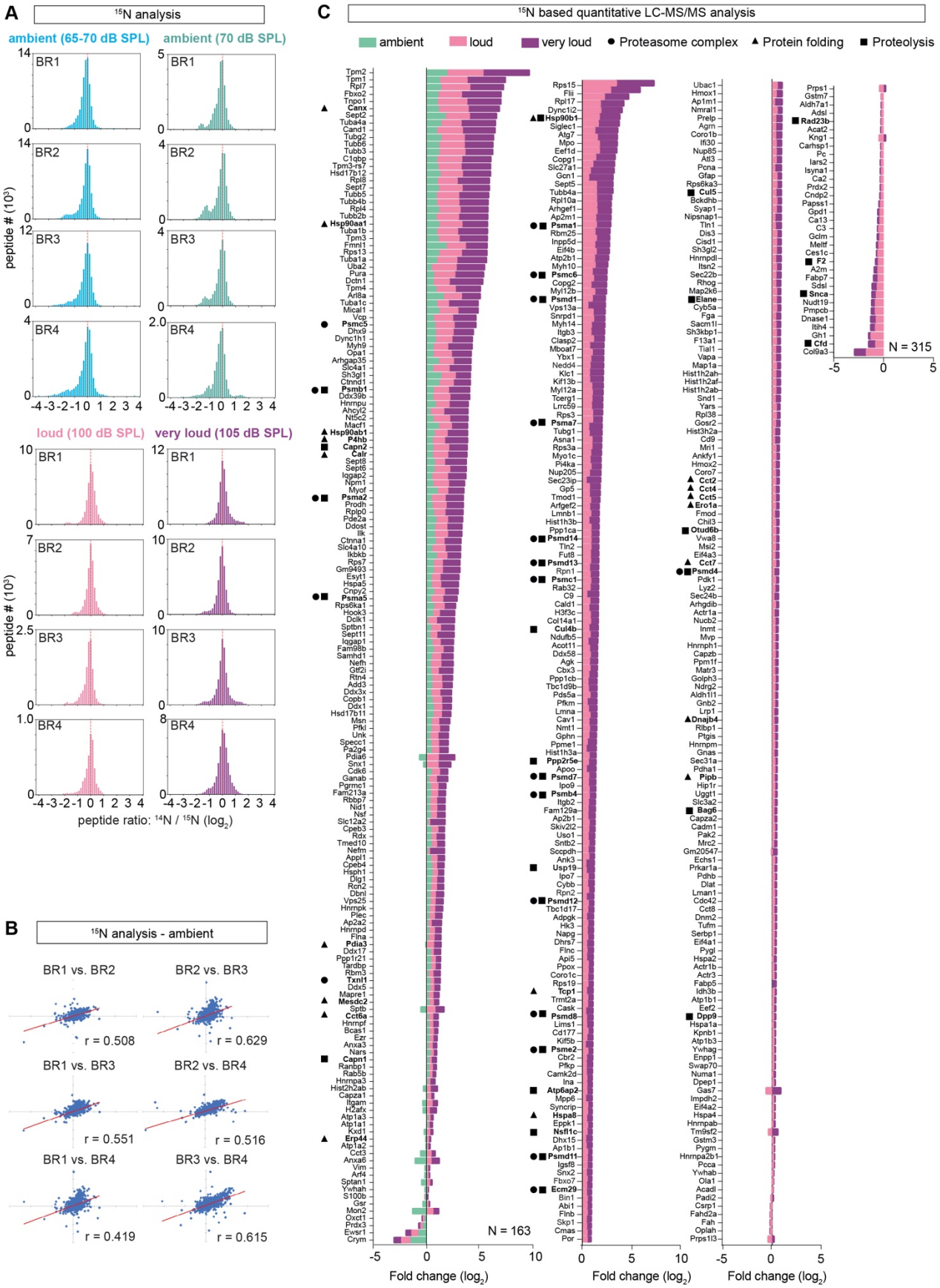

Figure S2 (related to Figure 1 and 2): Description of quantified proteins from ^{15}N LC-MS/MS analysis across noise exposures. (A) Normalized ^{14}N / ^{15}N peptide ratios from 4 biological replicates each exposed to ambient, loud, or very loud noise (65 – 70,

70, 100, or 105 dB SPL) for 30 minutes. Dotted red line indicates ^{14}N / ^{15}N = 1 (log_2 = 0). **(B)** Protein abundance correlation plots based on ^{14}N / ^{15}N ratios from 4 biological replicates exposed to ambient noise to determine the threshold log fold differences (TLFD) of regulated protein abundance. Linear regression analysis in each pair of correlation was plotted and multiple regression (r) was determined. **(C)** Representative significantly altered proteins (B.H. *p* value < 0.05) from cochlea exposure to ambient, loud and very loud noise. Majority of these proteins had elevated levels in a noise intensity dependent manner. Individual protein fold change are presented as stacked bar graphs. Proteostasis proteins based on GO analysis are indicated: ● = proteasome complex, ▲ = protein folding and ■ = proteolysis.

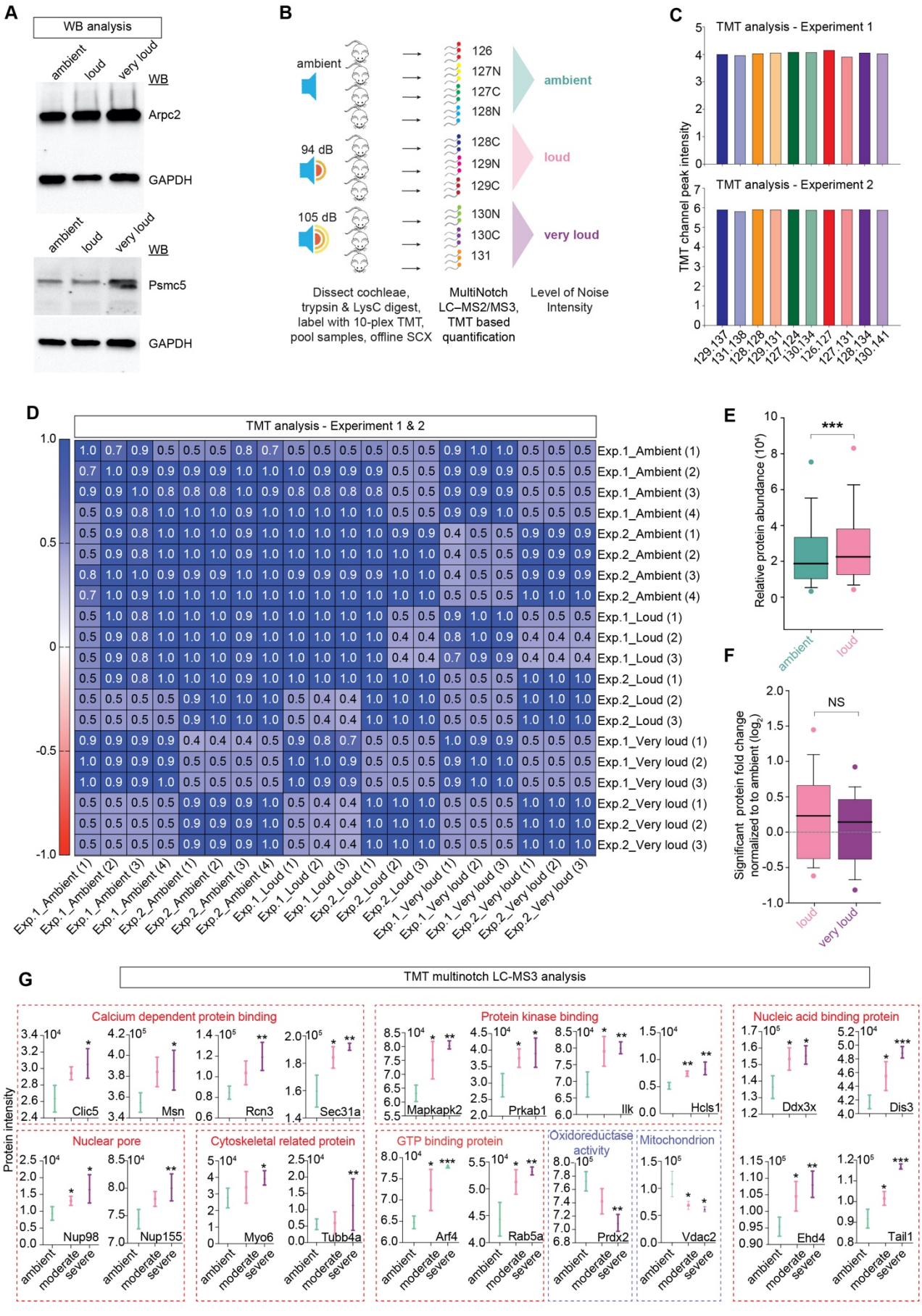
A
WB analysis
ambient
loud
very loud
WB
Arpc2
GAPDH
ambient
loud
very loud
WB
Psmc5
GAPDH
B
ambient
94 dB
105 dB
126
127N
127C
128N
128C
129N
129C
130N
130C
131
ambient
loud
very loud
Dissect cochleae, trypsin & LysC digest, label with 10-plex TMT, pool samples, offline SCX
MultiNotch LC–MS2/MS3, TMT based quantification
Level of Noise Intensity
C
TMT analysis - Experiment 1
TMT analysis - Experiment 2
TMT channel peak intensity
129.137
131.138
128.128
129.131
127.124
130.134
126.127
127.131
128.134
130.141
D
TMT analysis - Experiment 1 & 2
Exp.1_Ambient (1)
Exp.1_Ambient (2)
Exp.1_Ambient (3)
Exp.1_Ambient (4)
Exp.2_Ambient (1)
Exp.2_Ambient (2)
Exp.2_Ambient (3)
Exp.2_Ambient (4)
Exp.1_Loud (1)
Exp.1_Loud (2)
Exp.1_Loud (3)
Exp.2_Loud (1)
Exp.2_Loud (2)
Exp.2_Loud (3)
Exp.1_Very loud (1)
Exp.1_Very loud (2)
Exp.1_Very loud (3)
Exp.2_Very loud (1)
Exp.2_Very loud (2)
Exp.2_Very loud (3)
E
***
Relative protein abundance (10^4)
ambient
loud
F
NS
Significant protein fold change normalized to to ambient (log_2)
loud
very loud
G
TMT multinotch LC-MS3 analysis
Protein intensity
Calcium dependent protein binding
Clic5
Msn
Rcn3
Sec31a
Protein kinase binding
Mapkapk2
Prkab1
Ilk
Hcls1
Nucleic acid binding protein
Ddx3x
Dis3
Nuclear pore
Nup98
Nup155
Cytoskeletal related protein
Myo6
Tubb4a
GTP binding protein
Arf4
Rab5a
Oxidoreductase activity
Prdx2
Mitochondrion
Vdac2
Ehd4
Tail1
ambient
moderate
severe

Figure S3 (related to Figure 1-7): Assessment and confirmation of significantly altered proteins from TMT analyses. (A) Representative WB analysis of Arpc2 and Psmc5 from mice exposed to ambient, loud, or very loud noise for 30 minutes. **(B)** Analysis scheme to investigate noise exposures causing hearing loss acutely influence cochleae protein levels using 9 or 10plex tandem mass tags (TMT) relative quantitative proteomic analysis of mouse cochlear extracts. **(C)** Summary of individual TMT 10plex channel peak intensities prior to normalization. **(D)** Protein abundance correlation plots based on TMT ratios from 2 - 3 biological replicates exposed to ambient, loud, or very loud noise (65 – 70, 94, or 105 dB SPL). Each representative pair from ambient, loud, or very loud noise exposures were analyzed by linear regression. The correlation graphs were plotted and determined R-squared. **(E)** Summary box plot analysis for all quantified proteins from the ambient and loud noise exposure datasets (n = 3,691 proteins). The relative protein abundance on average was significantly elevated after exposure to noise at loud compared to ambient levels (2.87E3 $\pm$ 6.68E3, 3.28E3 $\pm$ 5.78E3, mean $\pm$ SD). Black bars indicate median. **(F)** Summary box plot analysis of significantly altered proteins (B.H. *p* value < 0.05) based on FC between loud and very loud noise exposure datasets normalized to ambient. The relative abundance of the significantly altered proteins was not significantly different between loud and very loud datasets relative to ambient (0.198 $\pm$ 0.665, 0.0437 $\pm$ 0.531, mean $\pm$ SD). Dotted line indicates protein FC = 0.0 **(G)** Selected TMT abundance plots for cytoskeletal, calcium depending protein binding, nuclear pore, nucleic acid binding, protein kinase binding, GTP binding, oxidoreductase, and mitochondrion associated proteins with significantly elevated or reduced levels across noise exposure for 30 minutes. *** = *p* value < 0.001 by Mann-Whitney (F), * = *p* value < 0.05, ** = *p* value < 0.01, *** = *p* value < 0.001 by one-way ANOVA with Bonferroni post hoc test (G). N = 1 mouse (A), 3-4 mice per group (B, E, F, G).

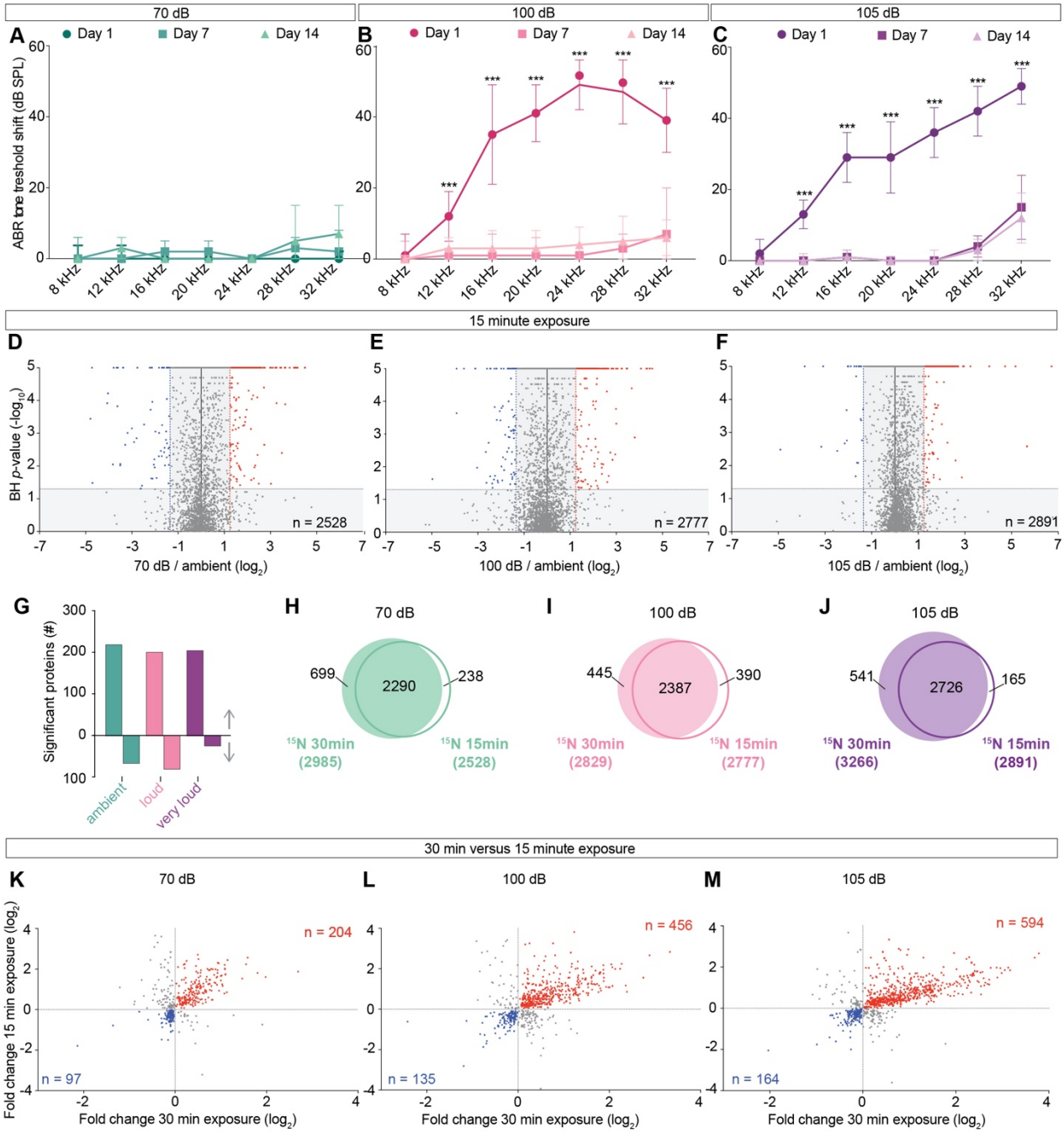

Figure S4 (related to Figure 1): Noise exposures causing hearing loss unbalance the cochlea proteome after 15 minutes of exposure to noise. (A-C) ABR tone recordings of FVB mice exposed to 70 dB SPL (**A**), 100 dB SPL (**B**), and 105 dB SPL (**C**) white band noise centered on the 8-16 kHz range for 15 minutes. ABR tone threshold levels were significantly elevated after 100 dB SPL by one-way ANOVA and 105 dB SPL conditions at day one compared to seven and fourteen days post noise exposure. (**D-F**) Volcano plots from ^{15}N based proteomic quantification of cochleae from mice expose to noise for 15 minutes at 70 dB SPL **(D)**, 100 dB SPL **(E),** and 105 dB SPL **(F),** graphed as log_2 fold change vs. $-log_{10}$ B.H. *p* value. Proteins that satisfied both the statistical (B. H. *p*-value < 0.05) and TLFD thresholds are in red (elevated) or blue (reduced). (**G**) Summary of the number of significantly regulated (B.H. *p* value < 0.05) proteins that reached the TLFD criteria. **(H-J)** Venn diagrams showing the overall number of quantified proteins in the 15 and 30-minute datasets. **(K-M)** Scatter plots comparing levels of significantly

altered proteins (B. H. *p* value < 0.05) in the 15 and 30 minute datasets. N > 8 mice (A-C), and 4 for (E-M).

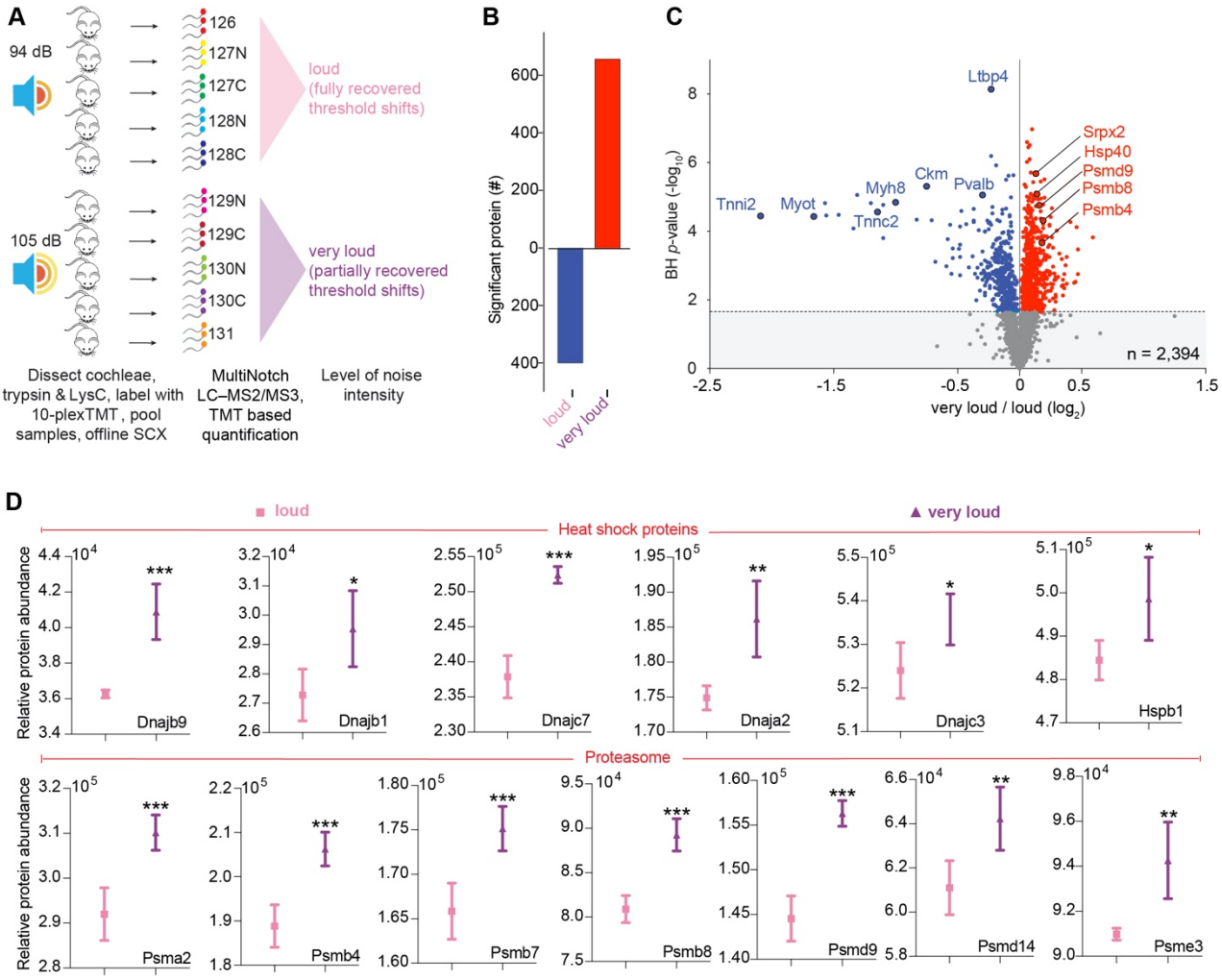

Figure S5 (related to Figure 2): Direct comparison of the cochlear proteome after 30-minute noise exposures causing temporary or permeant hearing loss. (A) Experimental scheme to determine how cochlear protein levels are acutely altered by noise exposures causing a TTS or a PTS with 10plex TMT. **(B)** Number of proteins with significantly (B.H. *p* value < 0.05) altered fold change. **(C)** Volcano plot depicting cochlear proteome remodeling after noise exposures causing TTS compared to PTS. Proteins meeting the statistical cutoff (B.H. *p* value < 0.05) are above the grey dotted line. Selected proteins with elevated levels are in red and reduced are in blue. **(D)** Selected TMT abundance plots for heat shock proteins and proteasome with significantly elevated or reduced levels between very loud and loud noise exposure for 30 minutes. * = *p* value < 0.05, ** = *p* value < 0.01, *** = *p* value < 0.001 by t test. N = 5 mice per group (B-D).

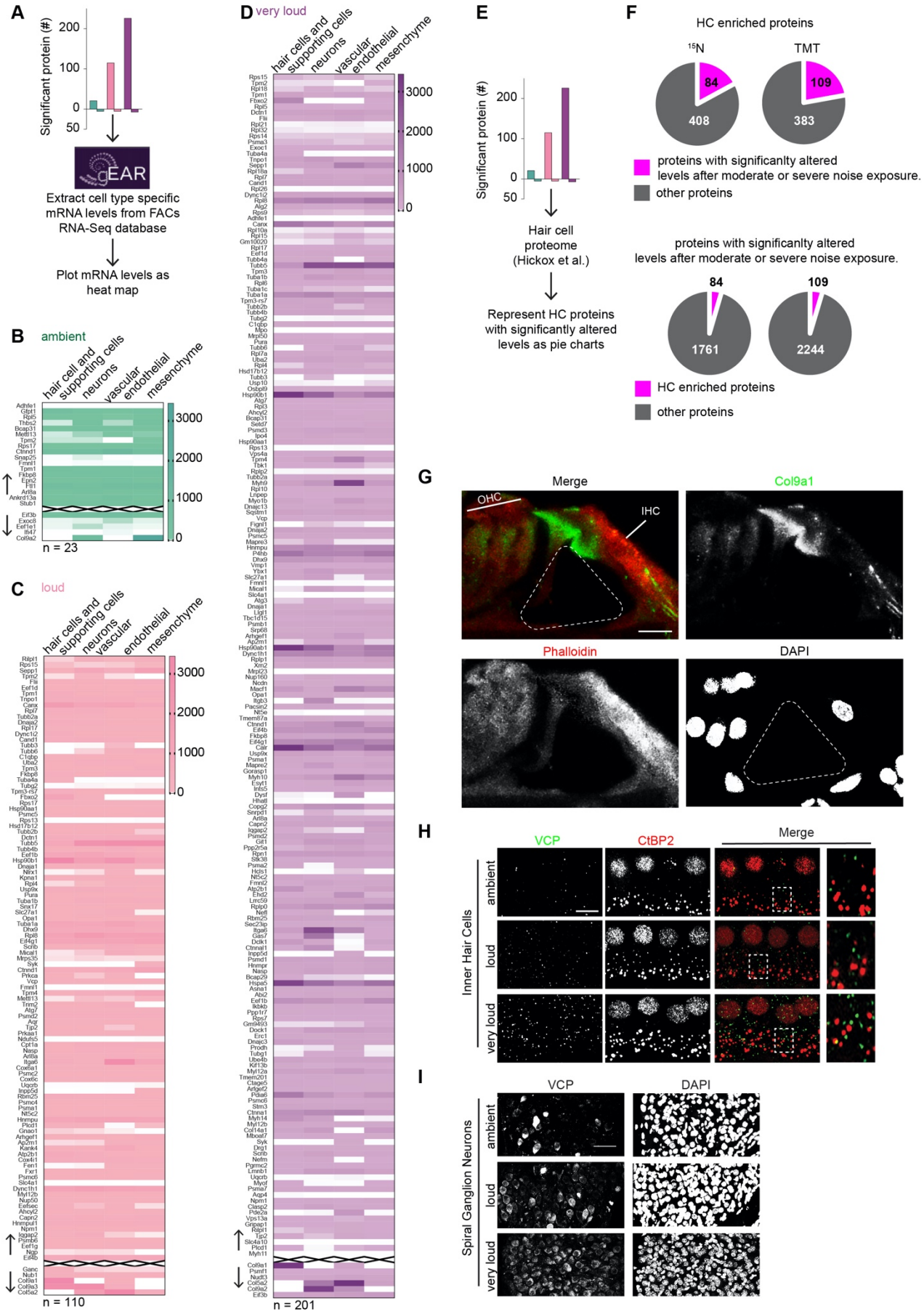
A
Significant protein (#)
200
100
0
50
gEAR
Extract cell type specific mRNA levels from FACs RNA-Seq database
Plot mRNA levels as heat map
B
ambient
hair cell and supporting cells
neurons
vascular
endothelial
mesenchyme
3000
2000
1000
0
n = 23
C
loud
n = 110
D
very loud
n = 201
E
Significant protein (#)
Hair cell proteome (Hickox et al.)
Represent HC proteins with significantly altered levels as pie charts
F
HC enriched proteins
15N
TMT
84
408
109
383
proteins with significanlty altered levels after moderate or severe noise exposure.
other proteins
proteins with significanlty altered levels after moderate or severe noise exposure.
84
1761
109
2244
HC enriched proteins
other proteins
G
Merge
Col9a1
OHC
IHC
Phalloidin
DAPI
H
VCP
CtBP2
Merge
Inner Hair Cells
ambient
loud
very loud
I
VCP
DAPI
Spiral Ganglion Neurons
ambient
loud
very loud

Figure S6 (related to Figure 4): Multiple cochlear cell types express proteins with altered levels after noise exposure. (A) Significantly altered proteins from the ^{15}N cochlear proteomic analysis of 30 minute noise exposures were mapped to genes within the gEAR inner ear gene expression database. Heat map depicts mRNA levels from microarray analysis of FACs sorted hair cell and supporting cells, neurons, vascular epithelia, and mesenchyme. **(B)** Gene expression patterns for proteins with significantly altered levels in the ambient (23 of 26 proteins mapped), **(C)** loud (110 of 121 proteins mapped), and **(D)** very loud intensity noise datasets (201 of 234 proteins mapped). (B-D) Arrows indicate proteins with elevated or reduced levels (separated by "xxxx"). **(E)** Identified proteins enriched in HCs by using our previous proteomic analysis of GFP expressing HCs isolated with fluorescence-activated cell sorting as a reference. **(F)** The comparison of proteins enriched in HCs to those found with significantly altered fold change after exposure to loud or very loud intensity noise from ^{15}N or TMT-based quantitative proteomics. **(G)** Col9a1 located at the area above tunnel of Corti which is the junction of inner and outer pillar cell. **(H-I)** VCP proteins levels are elevated during noise exposure and expressed by inner and outer hair cells **(H)** and spiral ganglion cells **(I)**. Scale bar = 10 μm (G and H) and 25 μm (I).

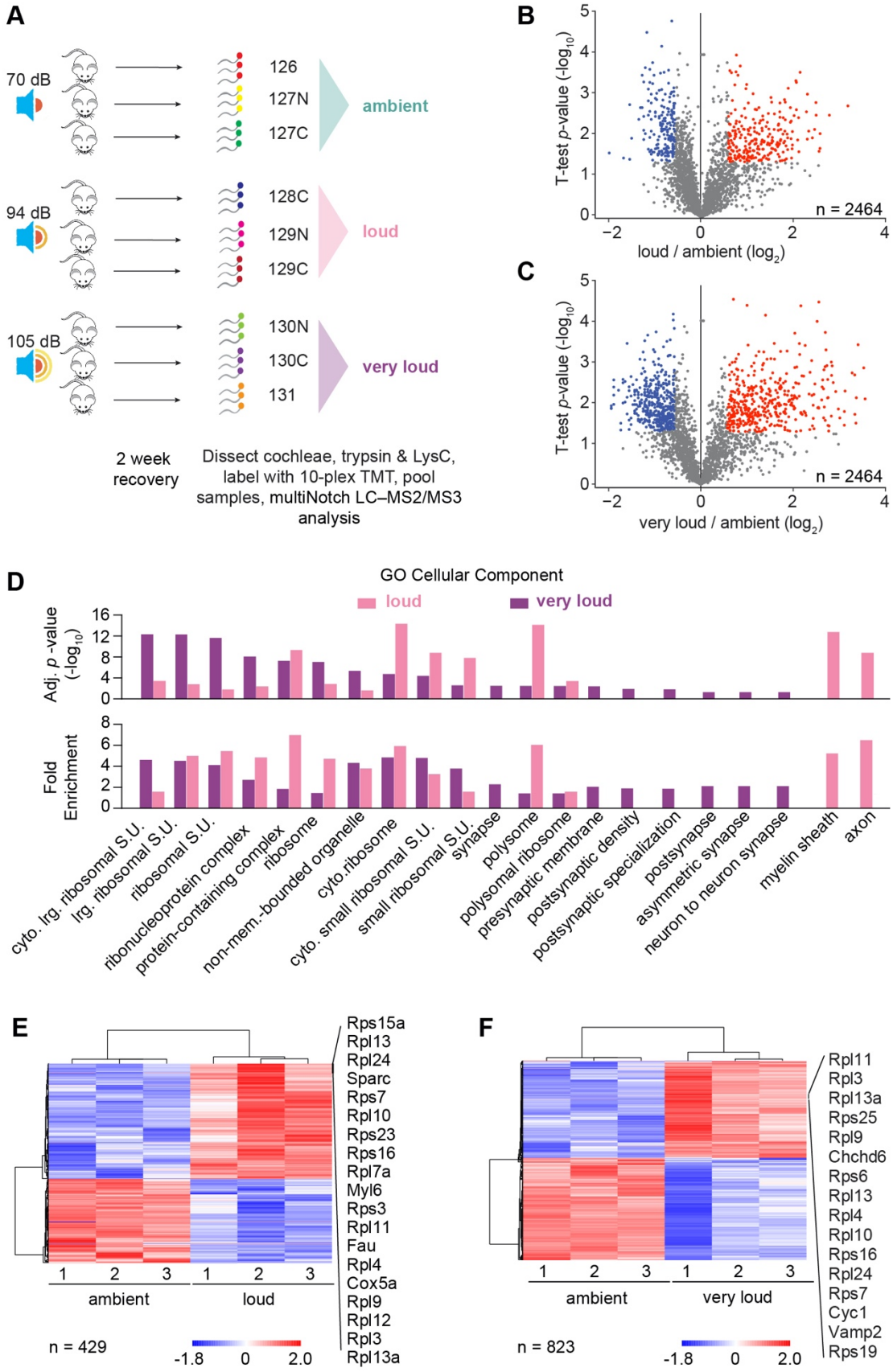

Figure S7 (related to Figure 7): Ribosomal proteins have elevated fold change during the hearing recovery period cochlear extracts after exposure to loud or very loud intensity noise relative to ambient intensity noise. (A) Quantitative 9plex TMT

proteomic analysis workflow to identify biological processes associated with the recovery phases of noise inducing temporary and permanent hearing loss relative to ambient controls. **(B)** Volcano plot depicting cochlear proteome remodeling two weeks after exposure to ambient versus loud noise for 30 minutes. Proteins meeting the statistical cutoff (T-test *p* value < 0.05) and having at least a 1.5 fold change are red (elevated) or blue (reduced). **(C)** Same as (B) except volcano plot depicts measures from very loud versus ambient intensity noise exposures. **(D)** GO: Cellular component enrichment analysis from the proteins with significantly altered fold change relative all the proteins identified. **(E and F)** Heatmaps depicting proteins with very loud intensity noise altered fold change for loud versus ambient (E) and very loud versus ambient (F) clustered based on one-minus correlation and average linkage. Ribosomal proteins robustly co-cluster in both datasets. N = 3 mice per group.