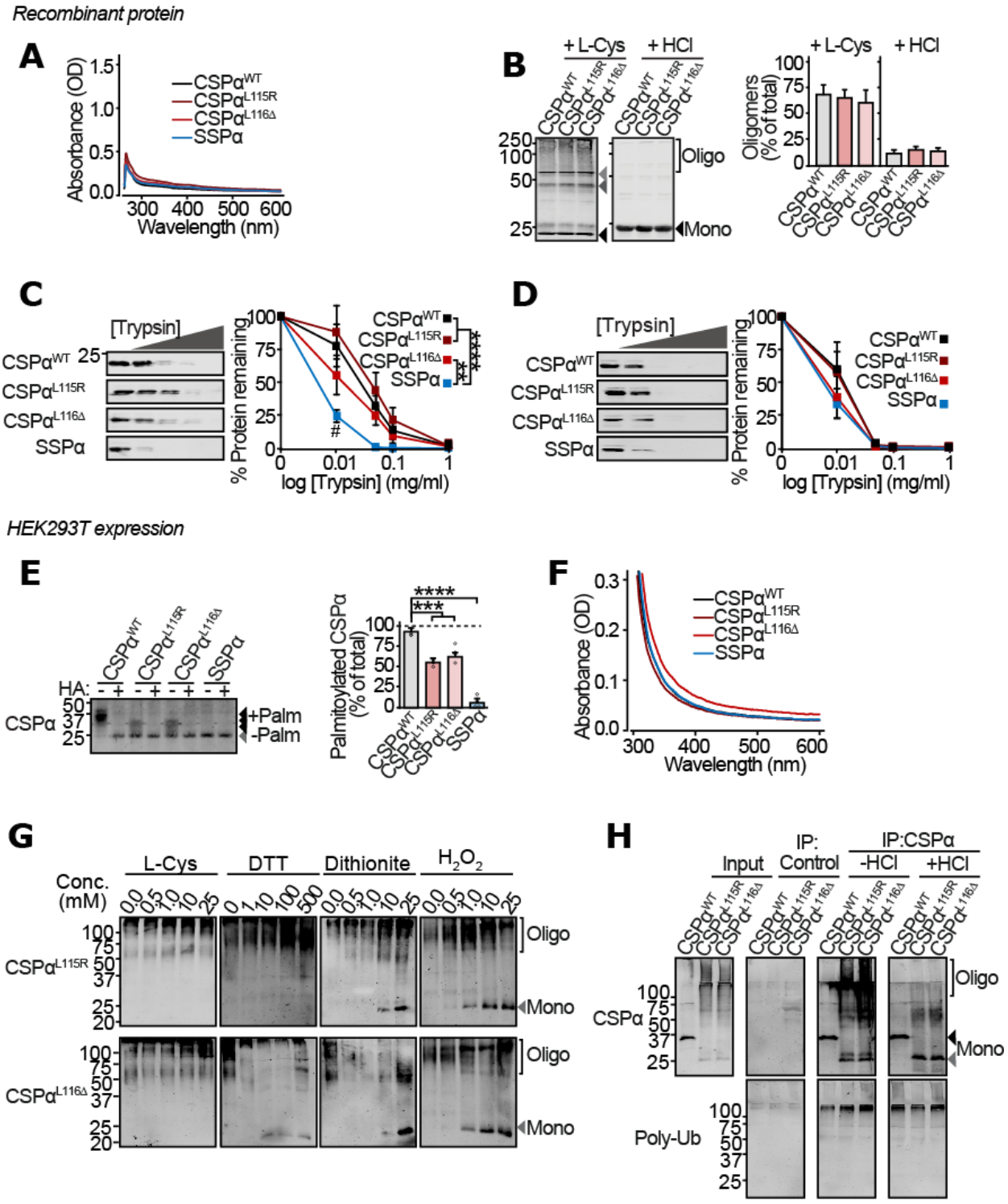
SUPPLEMENTARY FIGURES

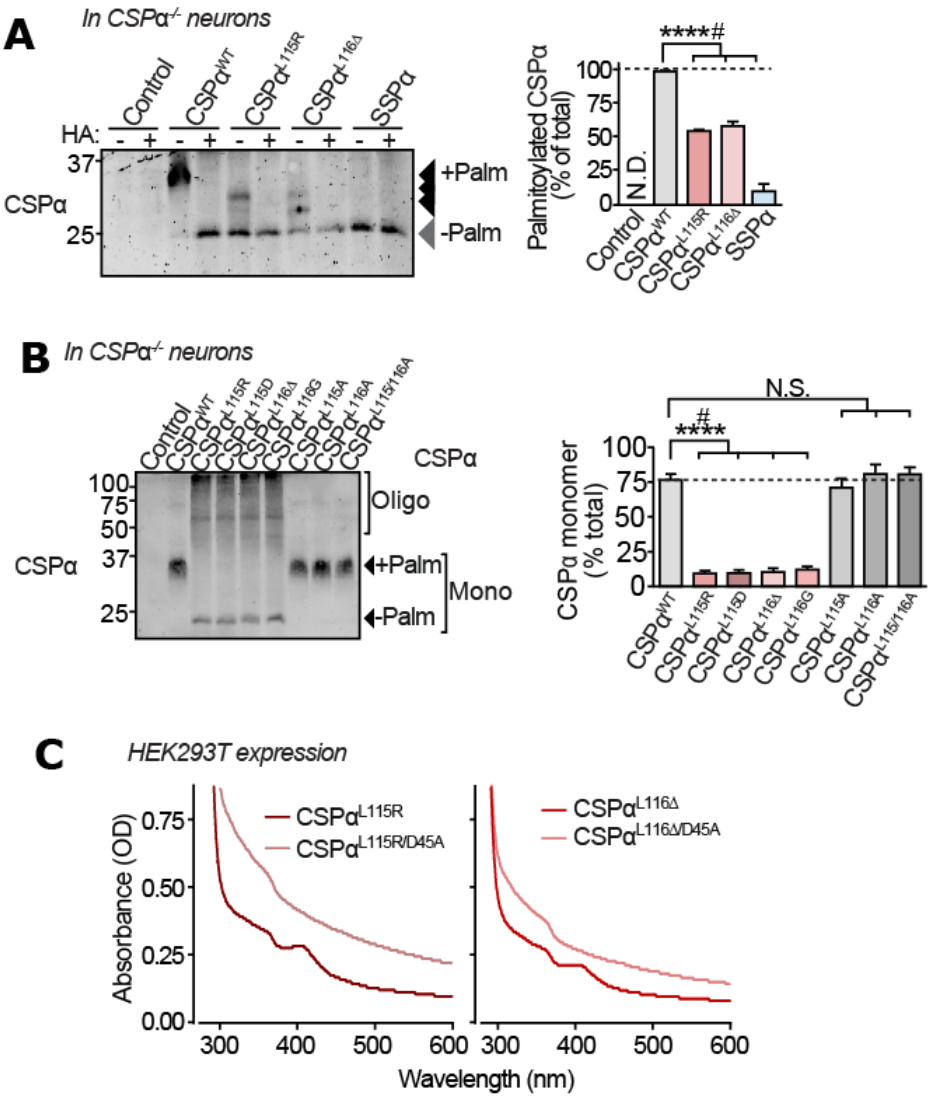
for Naseri et al.

“Aggregation of Mutant Cysteine String Protein-α via Fe-S Cluster-Binding is mitigated by Fe-chelators”



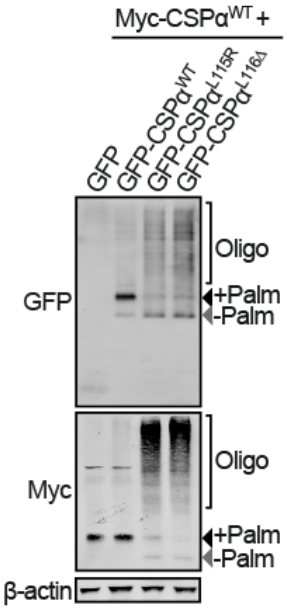
Supplementary Figure S1 | Fe-S cluster binding and oligomerization of CSPα *in vitro* and in mammalian cells. (**a-d**) Purified recombinant CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$ and SSPα: (**a**) UV-Vis absorbance spectra of purified proteins following HCl treatment. (**b**) Oligomers of CSPα were measured by quantitative immunoblotting following incubation with L-cysteine (L-Cys) or HCl. Mono = monomer; Oligo = oligomers; grey arrowheads = possible dimer and tetramer. (**c**) Purified proteinsα were subjected to increasing trypsin concentrations. CSPα oligomers were then disrupted with HCl,

followed by boiling in Laemmli sample buffer and quantitative immunoblotting against CSPα. (**d**) Same as in (c) except that before trypsinization, purified proteins were incubated with HCl . (**e-h**) Myc-tagged protein expression in HEK293T cells: (**e**) HEK293T cells transfected with $CSP\alpha^{WT}$, $CSP\alpha^{L115R}$, $CSP\alpha^{L116\Delta}$, or SSPα expressing vectors were lysed 48 h post-transfection in buffer containing HCl to disrupt any oligomerization mediated by Fe-S clusters. Neutralized lysates were treated with either Tris (-) or hydroxylamine (HA) and quantitatively immunoblotted against CSPα. Palm = palmitoylated. (**f**) UV-Vis absorbance spectra of immunoprecipitated proteins after HCl treatment. (**g**) Post-nuclear lysates from HEK293T cells expressing $CSP\alpha^{L115R}$ or $CSP\alpha^{L116\Delta}$ were incubated with L-cysteine, DTT, sodium dithionite or H_2O_2 at indicated concentrations, followed by SDS-PAGE and anti-CSPα immunoblotting. (**h**) Ubiquitination of the indicated CSPα versions was measured by immunoprecipitation of myc-CSPα followed by immunoblotting for co-immunoprecipitated polyubiquitin, with and without HCl treatment post-immunoprecipitation. In (**b, h**), any acid-denatured/aggregated protein was included in the loaded sample. Data are from (**a**) representative of n=3, (**b**) n=4 (L-Cys) and n=9 (HCl), (**c-d**) n=6, (**e**) n=3, (**f**) representative of n=3, (**g**) n=4. In (**a-d**) n = protein purification; in (**e-h**) n = transfection. Data represent means ± SEM. In (**b, e**) ***P<0.001; ****P<0.0001 by two-tailed Student's t-test, in (**c, d**) **P<0.01; ****P<0.0001 by two-way ANOVA excluding 1 mg/ml [trypsin], $^{\#}P \leq 0.05$ by Tukey's multiple comparisons test.



Supplementary Figure S2 | Reduced palmitoylation and Fe-S cluster binding of ANCL-causing and artificial CSPα mutants. (**a-b**) Primary cortical neurons from neonatal CSPα$^{-/-}$ mice were lentivirally infected with indicated version of CSPα on 7 days *in vitro* (DIV), and harvested on 15 DIV. (**a**) Neurons expressing myc-tagged CSPα^{WT}, CSPα^{L115R}, CSPα$^{L116\Delta}$, SSPα, or empty virus (control) were lysed in 0.1 N HCl containing 0.1% Triton X-100 for 20 min at 4 °C, to disrupt any oligomerization mediated by Fe-S clusters. Lysate was neutralized with 1 M sodium cacodylate to pH 7.0 and treated for 16 h with either 0.5 M Tris (-) or with 0.5 M hydroxylamine (HA) at 4 °C, followed by quantitative immunoblotting against CSPα. Depalmitoylation (HA) illustrates palmitoylation-dependent mass shift. (**b**) Neurons expressing myc-tagged CSPα^{WT}, CSPα^{L115R}, CSPα^{L115D}, CSPα$^{L116\Delta}$, CSPα^{L116G}, CSPα^{L115A}, CSPα^{L116A}, or CSPα$^{L115/116A}$ were lysed in non-reducing Laemmli sample buffer. Monomer levels of indicated CSPα variants were measured by SDS-PAGE separation and quantitative immunoblotting. Representative immunoblot (left), and quantitation (right) as % of the total protein detected in corresponding lane. Mono = monomer; Oligo = oligomers; Palm = palmitoylated. (**c**) From

HEK293T cells expressing myc-tagged CSPα^{L115R}, CSPα$^{L115R/D45A}$, CSPα$^{L116\Delta}$, and CSPα$^{L116\Delta/D45A}$. UV-Vis absorbance spectra of indicated proteins immunoprecipitated using anti-myc antibody and eluted by trypsinization. Data are from (**a**) n=5 transductions, (**b**) n=4 transductions, and (**c**) representative spectra from n=3 independent CSPα immunoprecipitations. In (**a-b**) data represent means ± SEM. ****P<0.0001; N.S. = not significant by two-tailed Student's t-test; $^{\#}$P<0.05 by Mann-Whitney-Wilcoxon *U* test.



Supplementary Figure S3 | Mutant CSPα draws wild type CSPα into aggregates. CSPα$^{-/-}$ primary cortical neurons were lentivirally transduced to co-express myc-CSPα^{WT} with either GFP-CSPα^{WT}, GFP-CSPα^{L115R}, or GFP-CSPα$^{L116\Delta}$ (control = GFP alone). Shown is one representative experiment from n=3 independent co-transductions. Oligo = oligomerized; Palm = palmitoylated.

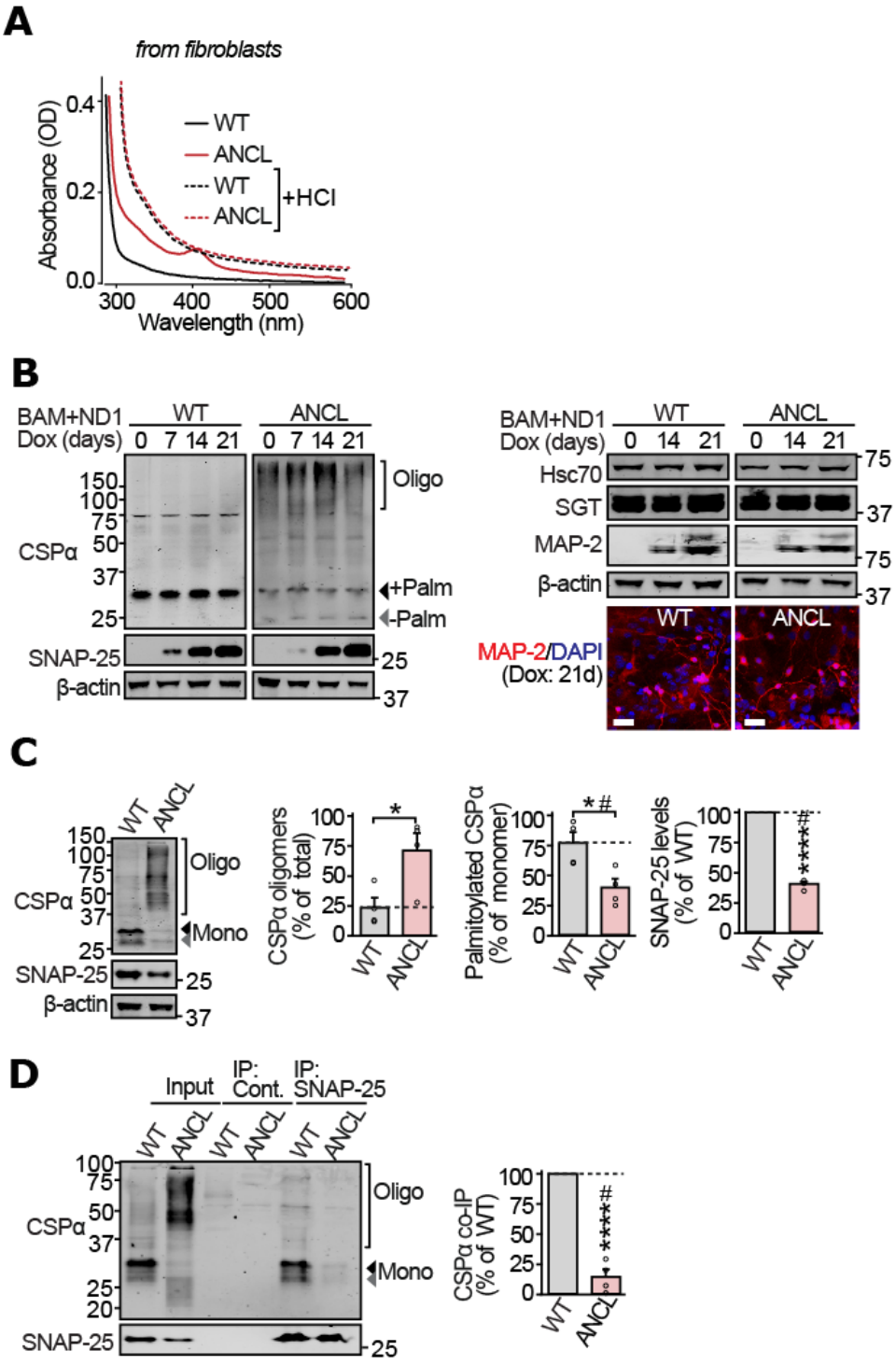
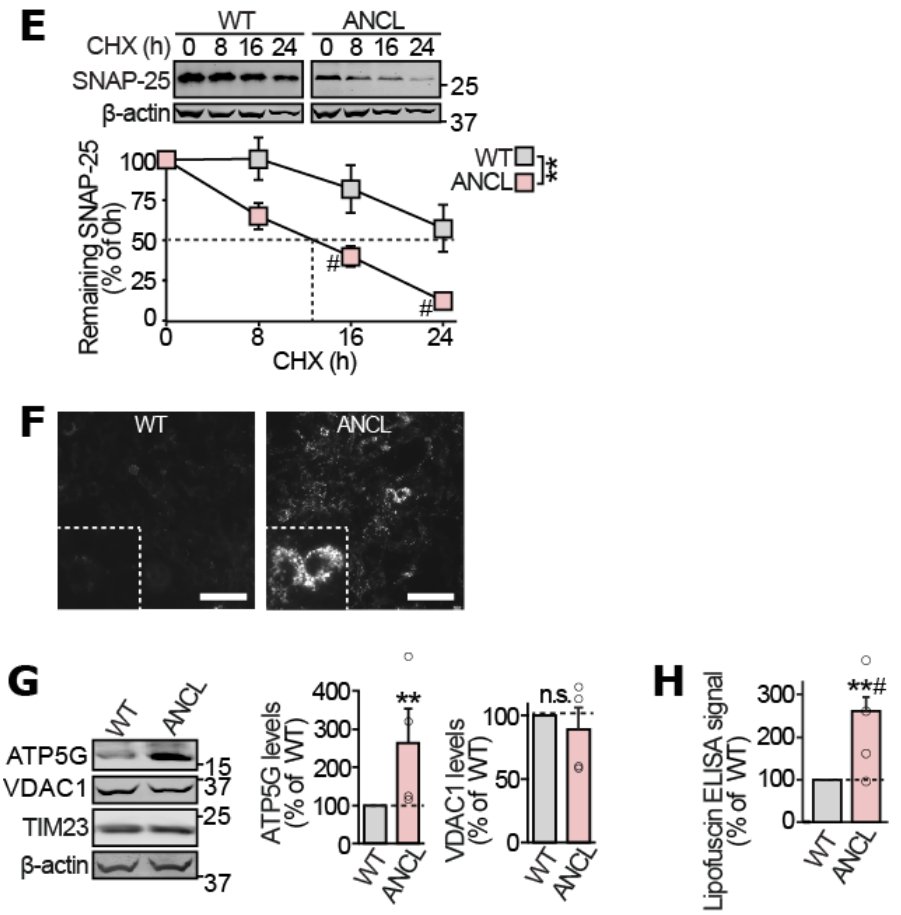


Fig. S4 Continues on next page.



Supplementary Figure S4 | ANCL patient-derived fibroblasts and iNs reveal CSPα defects with downstream SNAP-25 instability and lipofuscin accumulation. (**a**) UV-Vis absorbance spectra of immunoprecipitated CSPα from WT and ANCL patient fibroblasts, before (solid lines) and following (dashed lines) 0.1 N HCl incubation for 20 min at R/T (dashed lines). (**b**) Time course of direct induction of WT and ANCL fibroblasts into iNs using BAM+ND1 expression. Immunoblots show the expression of neuronal marker MAP-2 and synaptic SNARE SNAP-25, as well as that of CSPα and its chaperone complex partners Hsc70 and SGT. Anti-MAP2 immunofluorescence following 21 d of induction (bottom panels). Dox = doxycycline; scale bar = 50 μm. (**c**) CSPα oligomerization, palmitoylation and SNAP-25 levels were measured in WT and ANCL iNs by quantitative immunoblotting. (**d**) Interaction of CSPα SNAP-25 was measured in WT and ANCL iNs by immunoprecipitating SNAP-25 followed by quantitative immunoblotting of co-immunoprecipitated CSPα (normalized to the immunoprecipitated SNAP-25). Cont. = control sham IP without IgG. (**e**) Turnover of SNAP-25 was determined in WT and ANCL iNs by cycloheximide (CHX) chase followed by quantitative immunoblotting. (**f-h**) WT and ANCL iNs were analyzed at 56 days following BAM+ND1 induction protocol for (**f**) lipofuscin autofluorescence at Ex 465-495, Em 515-555 nm range (bar = 50 μm), (**g**) total levels of ATP5G accumulation by quantitative immunoblotting (VDAC1 and TIM23 indicate overall mitochondrial protein expression), and (**h**) lipofuscin accumulation by ELISA. Data are from (**a**) representative of n=3 immunoprecipitations; (**b**) representative of n=3 iN conversions; (**c**) n=4 iN conversions; (**d**) n=4

immunoprecipitations; (**e**) n=6 for WT and n=5 for ANCL (n = iN conversions); (**f**) representative of n=3 iN conversions; (**g-h**) n=4 iN conversions. In (**c-d**) and (**g-h**) *P<0.05; **P<0.01; ***P<0.001; ****P<0.0001 by two-tailed Student's t-test; #P<0.05 by Mann-Whitney-Wilcoxon *U* test. In (**e**) **P<0.01 by two-way ANOVA; #P<0.05 by Tukey's multiple comparisons test.