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Supplemental information

De novo serine biosynthesis is protective in mitochondrial disease

Christopher B. Jackson, Anastasiia Marmyleva, Geoffray Monteuuis, Ryan Awadhpersad, Takayuki Mito, Nicola Zamboni, Takashi Tatsuta, Amy E. Vincent, Liya Wang, Nahid A. Khan, Thomas Langer, Christopher J. Carroll, and Anu Suomalainen

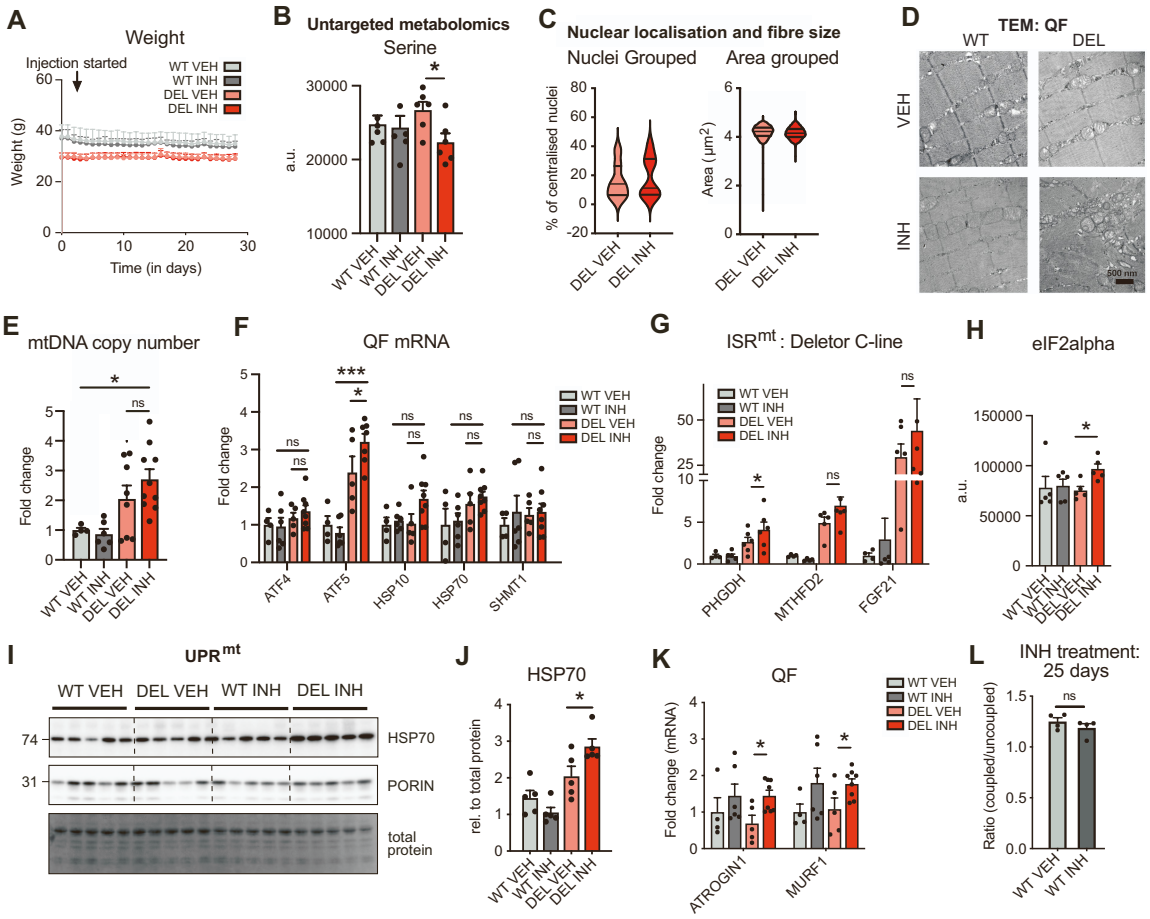

Figure S1 (related to Figure 1): Effects of inhibiting de novo serine biosynthesis (dnSB) via NCT-503 in mice.

(**A**) Weight change over time of treatment with NCT-503 (WT VEH n=6, Del VEH n=5, WT INH n=6, Del INH n=8).

(**B**) Serine level upon PHGDH inhibition. NCT-503 administration for 30 days; untargeted metabolomics of skeletal muscle (WT VEH n=6, Del VEH n=6, WT INH n=6, Del INH n=8). a.u. – arbitrary units.

(**C**) Quantification of muscle cross section fibre area and nuclear localization from Fig. 1F. Intensities normalized and expressed in a.u. (arbitrary units).

(**D**) Electron microscopic analysis. Right panels show distorted mitochondria in vehicle- and inhibitor-treated Deletor muscles.

(**E**) Mitochondrial DNA copy number analysis, qPCR (n=5-10/group).

(F) Transcriptional upregulation of components of stress response (ATF4, ATF5, HSP10, HSP70/GRP75, SHMT1). qPCR (n=4-6/group).

(**G**) Transcriptional upregulation of ISR^{mt} markers PHGDH, MTHFD2 and FGF21 in NCT-503 treated C-line Deletors. qPCR (n=4-6/group).

(**H**) Quantification of immunoblot of phosphorylated eIF2α / total eIF2α (n=5/group) from Fig. 1J.

(**I**) Immunoblot of stress marker HSP70/GRP75 and mitochondrial mass marker. Whole muscle QF lysate.

(**J**) Quantification of HSP70 relative to total protein (n=5/group) from (I).

(**K**) Transcriptional levels of muscle atrophy markers ATROGIN1 and MURF1. qPCR (n=5-8/group).

(**L**) Ratio of coupled and uncoupled respiration in mitochondrial-enriched QF fractions of 25-days untreated and NCT-503 treated WT mice (n=4 per group).

Error bars represent SEM. Statistical significance was assessed using ANOVA, * p≤0.05, **p≤0.01. ***p≤0.001.

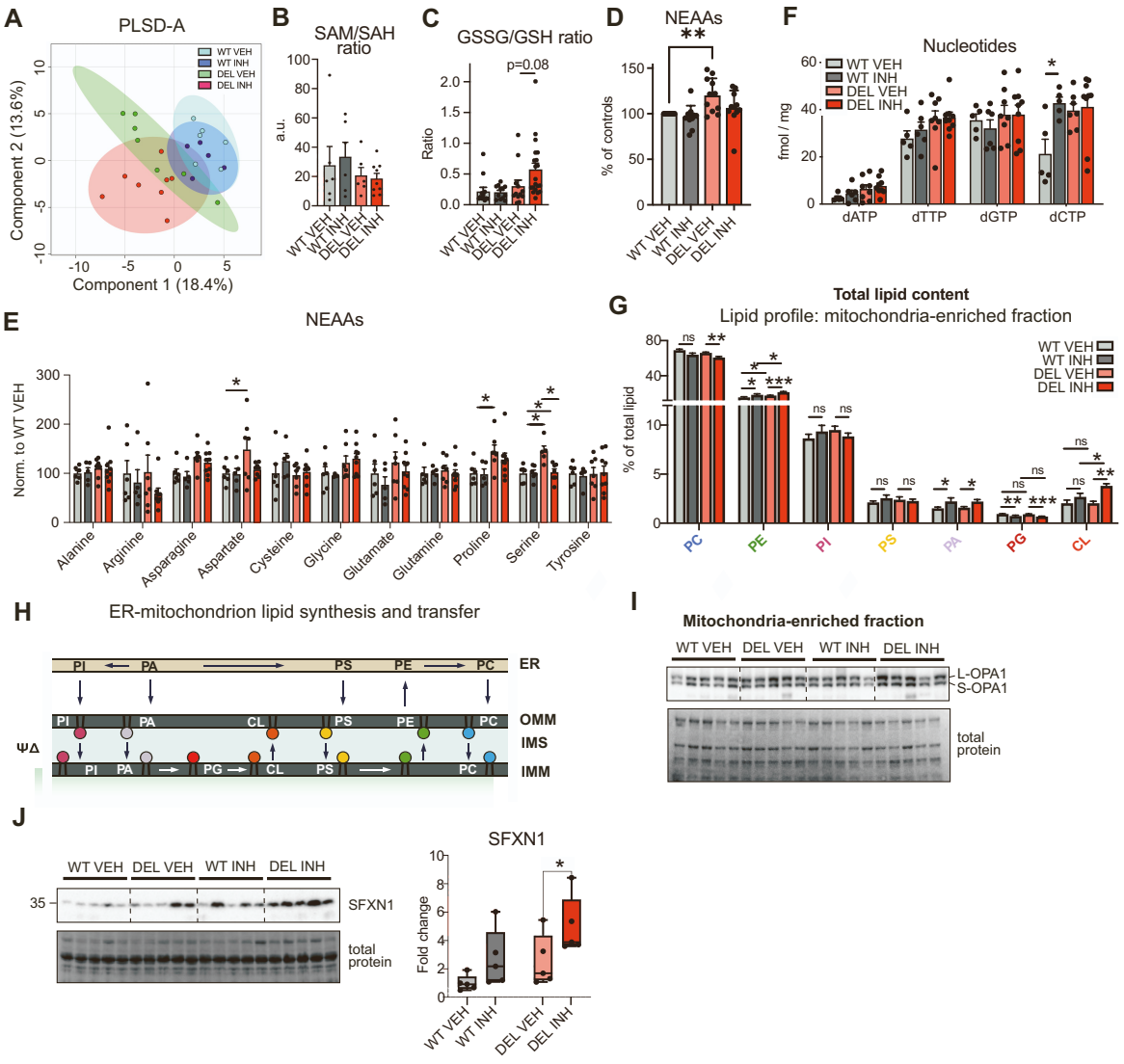

Figure S2 (related to Figure 2): Effect of dnSB inhibition via NCT-503 on muscle metabolome and structural markers.

(**A**) PLS-DA plot of targeted metabolomics from skeletal muscle (WT VEH n=6, Del VEH n=5, WT INH n=7, Del INH n=8).

(**B**) SAM/SAH metabolic ratio from Fig. 2A.

(**C**) Ratio of oxidized (GSSG) and non-oxidised glutathione from Fig. 2A.

(**D**) Total non-essential amino acid quantification (NEAAs) from Fig. 2A.

(**E**) Individual non-essential amino acid quantification (NEAAs) from Fig. 2A.

(**F**) Total nucleotide (dNTP) pools in skeletal muscle (WT VEH n=5, Del VEH n=7, WT INH n=6, Del INH n=8).

(**G**) Lipid content in mitochondria-enriched fraction (WT VEH n=5, Del VEH n=7, WT INH n=6, Del INH n=8). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidic acid (PA),

phosphatidylglycerol (PG) and cardiolipin (CL).

(**H**) Mitochondrial lipid synthesis schematic (adapted from Mesmin, 2016).

(**I**) Immunoblot of mitochondria-enriched fraction from skeletal muscle for OPA1 (n=5/group).

(**J**) Immunoblot of total muscle for SFXN1 with quantification (n=5/group)

Bars represent group average and error bars – standard error of mean (SEM). Statistical significance was assessed using ANOVA, * p≤0.05, **p≤0.01. ***p≤0.001.

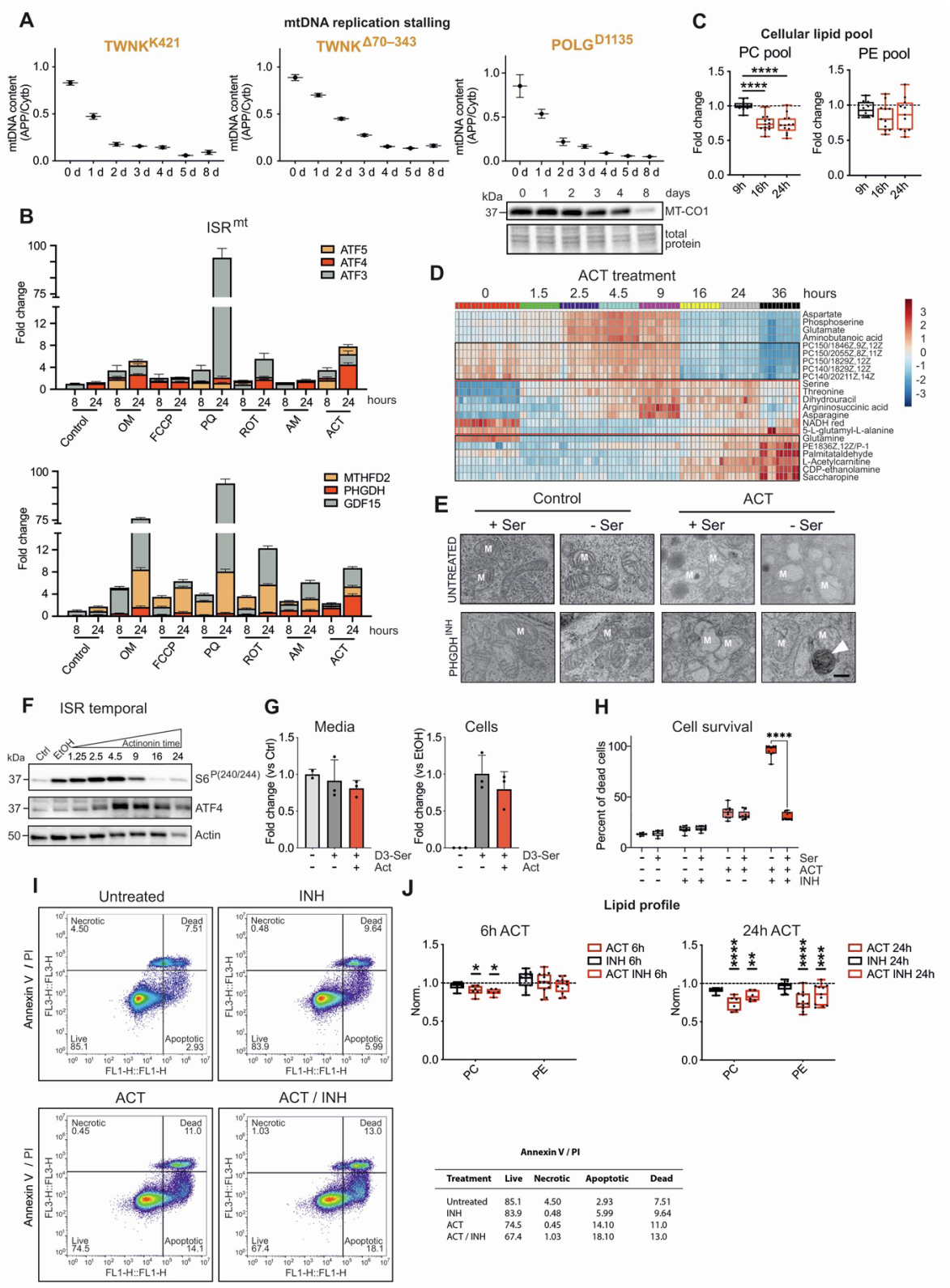

Figure S3 (related to Figure 3): Effect of temporal mitochondrial dysfunction and dnSB inhibition or serine deprivation.

(**A**) MtDNA copy number dynamics upon overexpression of catalytic Twinkle (TWNK)- and DNA-polymerase gamma (POLG) mutants (TWNK^{K421A}, TWNK$^{\Delta 70–343}$,

POLG^{D1335A} [43]); qPCR analysis. HEK293T cells. POLG^{D1135A} mutant overexpression in HEK293T cells shows gradual decrease in mtDNA copy number with concomitant but lagging loss of MT-CO1. Y axis represents days of mutant expression.

(**B**) Transcript levels of associated ISR^{mt} genes upon treatment with mitochondrial toxins for 8 and 24 hours. Human myoblasts. qPCR.

(**C**) Total lipid pools including PC and PE species of actinonin-treated C2C12 cells for the indicated time; untargeted temporal metabolomics. Each dot represents the median of a lipid species.

(**D**) Heatmap of selected top significantly altered metabolites in human myoblasts temporally treated with actinonin; untargeted metabolomics.

(**E**) Electron microscopy analysis of human myoblasts exposed to a combination of stress factors: serine availability (+/- SER), inhibition of mitochondrial translation (ACT) and/or de novo serine biosynthesis (PHGDH^{INH}). M – mitochondria, white arrow – lipid aggregate.

(**F**) Immunoblot of temporal actinonin treatment for ATF4 and mTOR target, phospho-S6 (p-S6).

(**G**) D^3-serine uptake. Amount in media and in C2C12 cells; biological replicates (n=3).

(**H**) Cell survival in C2C12 under inhibition of mitochondrial translation (ACT) and/or de novo synthesis (INH) in combination of serine-containing or deprived (+/- SER) conditions.

(**I**) C2C12 cell viability in serine-containing media. FACS (Annexin V / PI).

(**J**) Normalised PC and PE pools in C2C12 cells challenged with disrupted mitochondrial translation (ACT) and/or de novo serine synthesis (INH); untargeted metabolomics. Significance calculated against untreated control.

Error bars represent standard deviation (SD). Statistical significance was assessed using ANOVA, * p≤0.05, **p≤0.01. ***p≤0.001. Abbreviations: PHGDH=D-3-phosphoglycerate dehydrogenase; ACT= actinonin, INH= NCT-503.

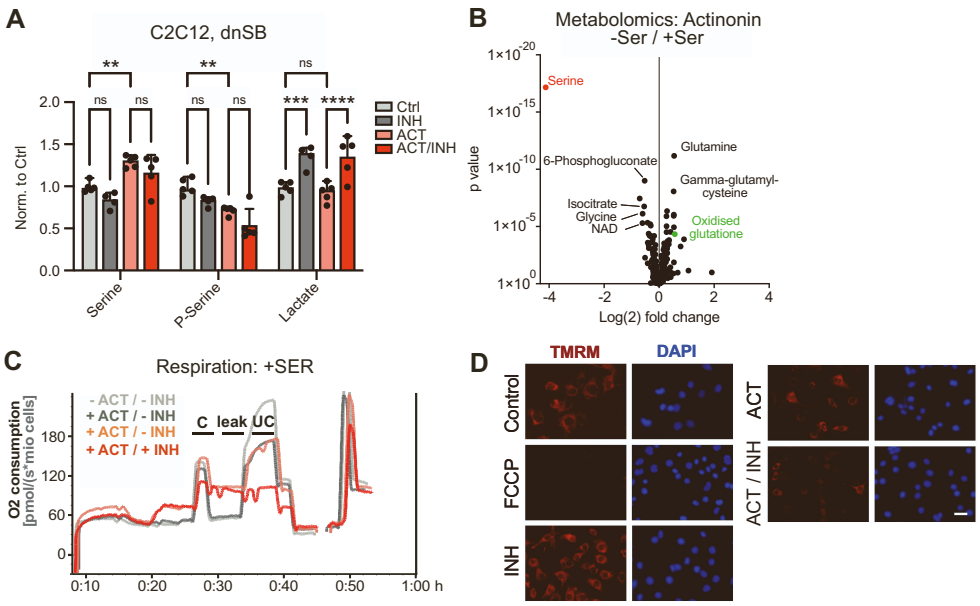

Figure S4 (related to Figure 3): Effects of dnSB inhibition on metabolites, oxidative function and mitochondrial membrane potential.

(**A**) Relative expression of serine, phosphoserine (P-serine) and lactate in C2C12 cells after treatment with actinonin and/or NCT-503; related to Fig. 3F.

(**B**) Effects of short (2.5 h) inhibition of mitochondrial translation in C2C12 cells in media with or without serine compared to actinonin-only treated cells, untargeted metabolomics. Metabolic data points represent the mean of biological quintuplicates.

(**C**) Respiration traces in permeabilised C2C12 cells grown in actinonin and/or under PHGDH inhibition. C = coupled, UC = uncoupled.

(**D**) Mitochondrial membrane potential upon inhibition of mt-translation and/or *de novo* serine biosynthesis. TMRM-probe detection, DAPI for cell count, C2C12 cells. Scale bar: 40uM. Quantification in Fig. 3L.

Error bars represent standard deviation (SD). Statistical significance was assessed using ANOVA, * p≤0.05, **p≤0.01. ***p≤0.001. Abbreviations: PHGDH=D-3-phosphoglycerate dehydrogenase; ACT= actinonin, INH= NCT-503.

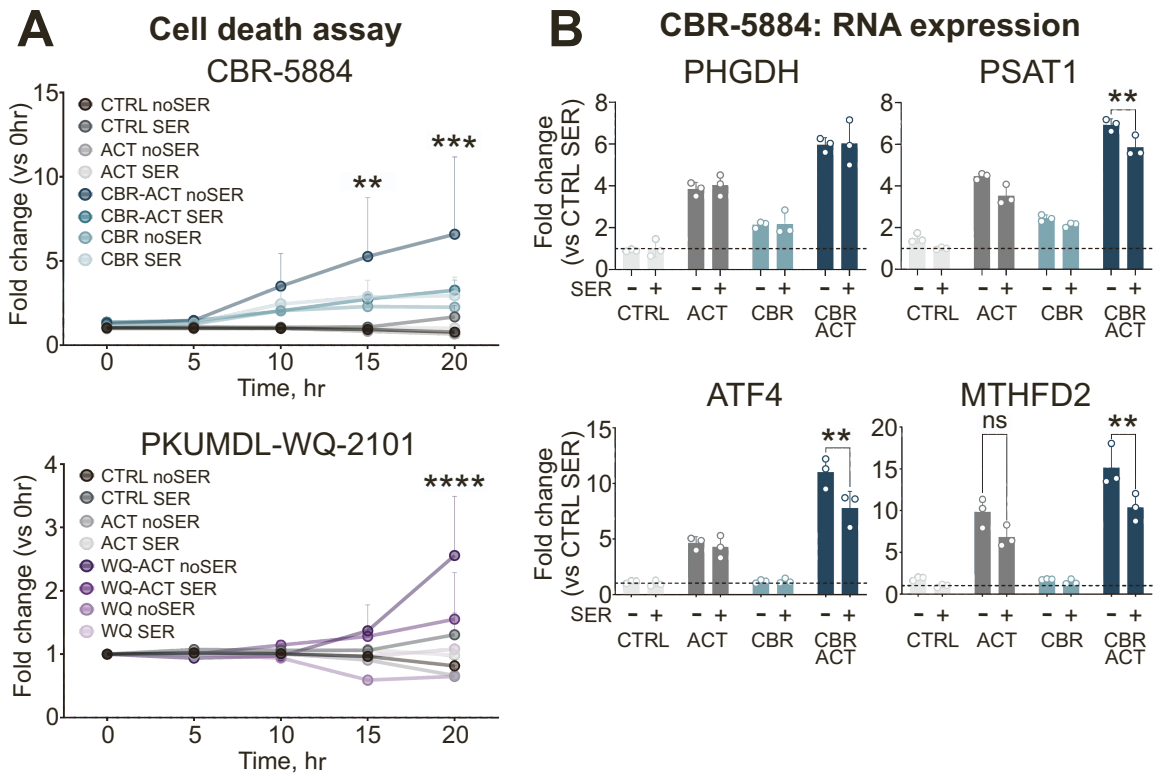

Figure S5 (related to Figure 3): Effect of dnSB inhibition and serine inhibition in cells.

(**A**) Cell death counts in response to PHGDH pharmacological inhibitors (CBR-5884, PKUMDL-WQ-2101) and/or actinonin. C2C12 cells in DMEM media with/without serine. Statistical comparisons between no serine (noSER) and serine (SER) containing media. n=3.

(**B**) Transcriptional expression of ISR^{mt} markers (PHGDH, PSAT1, ATF4 and MTHFD2) in response to treatments with CBR-5884 and/or actinonin. C2C12 cells in DMEM media with/without serine. qPCR; n=3.

Error bars represent SD. Statistical analysis: two-way ANOVA with Tukey correction for multiple comparisons.