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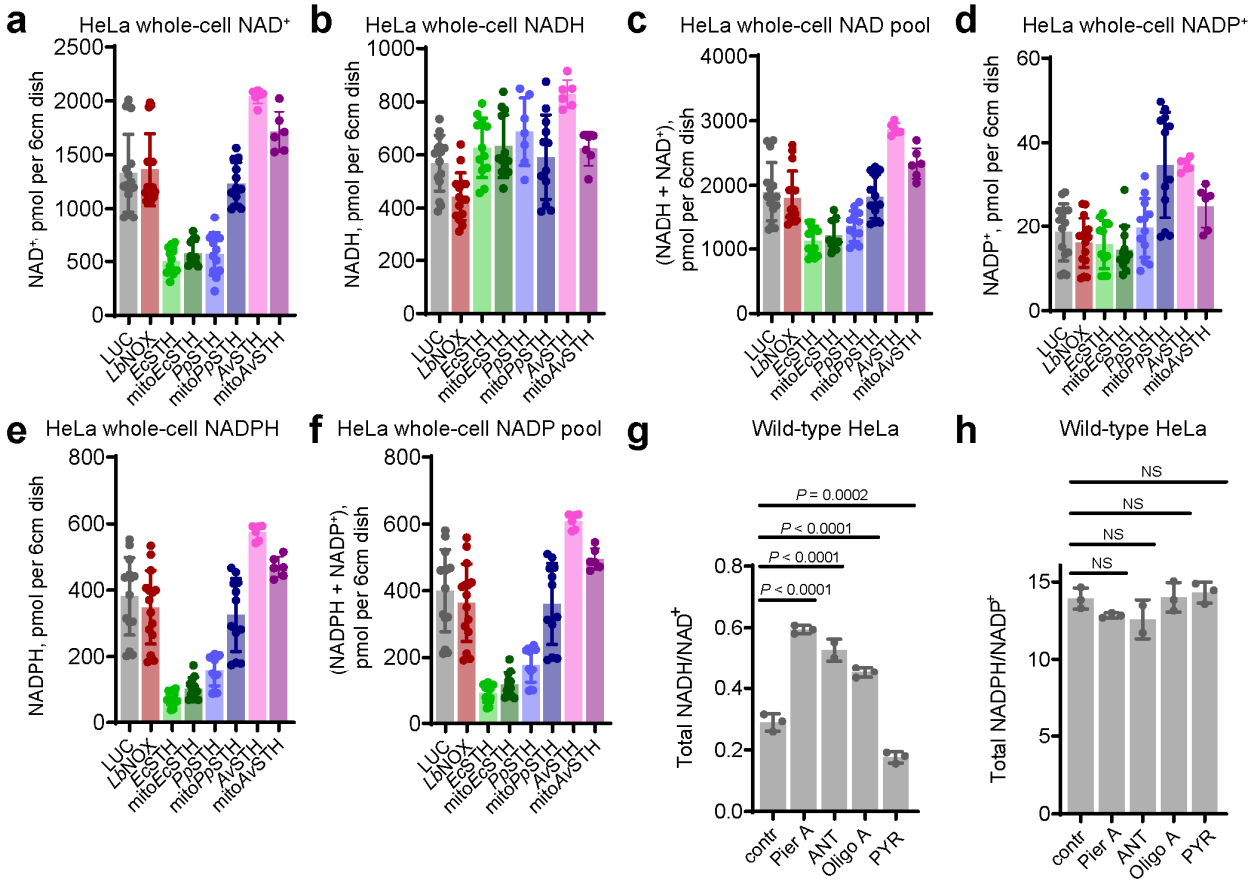
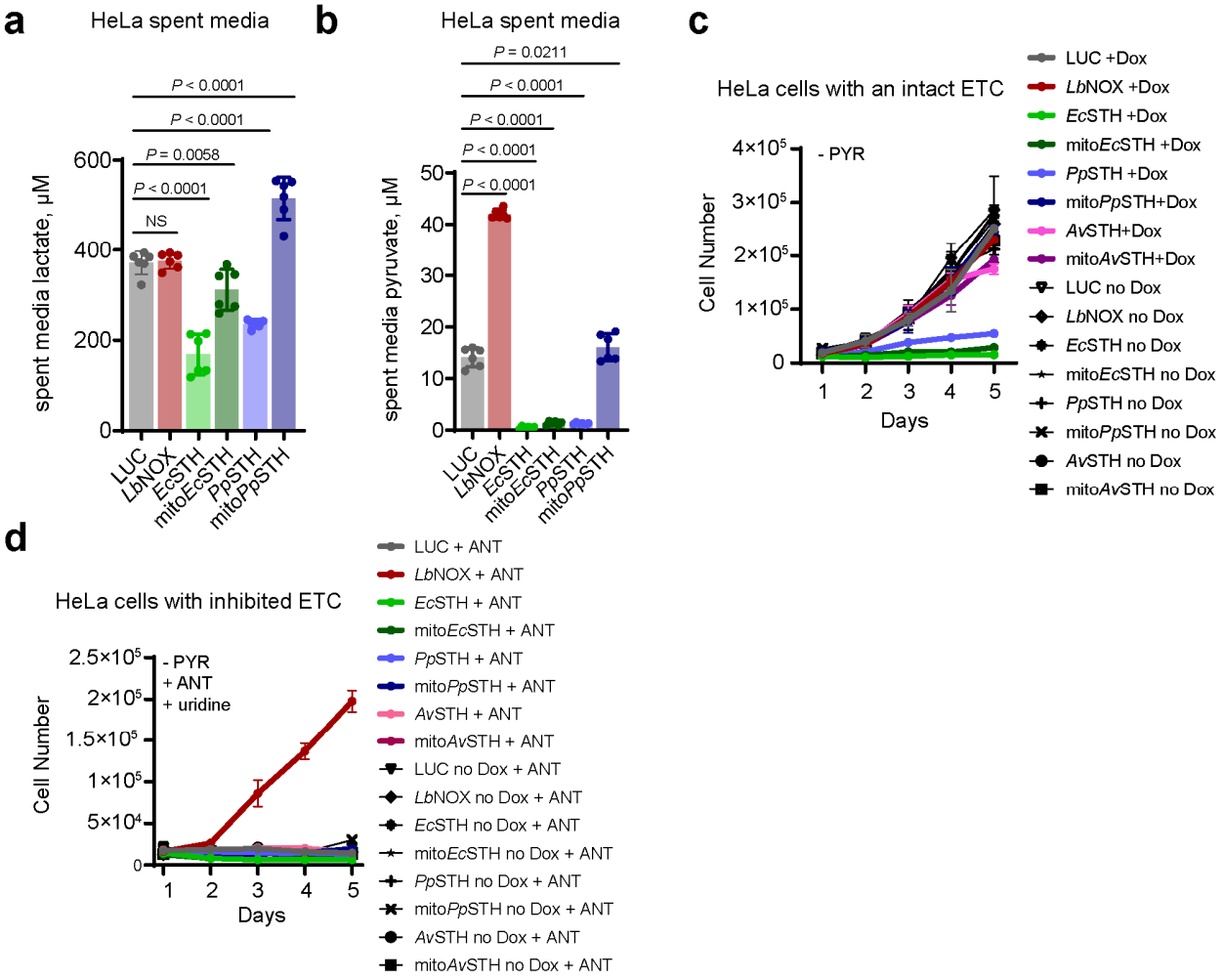
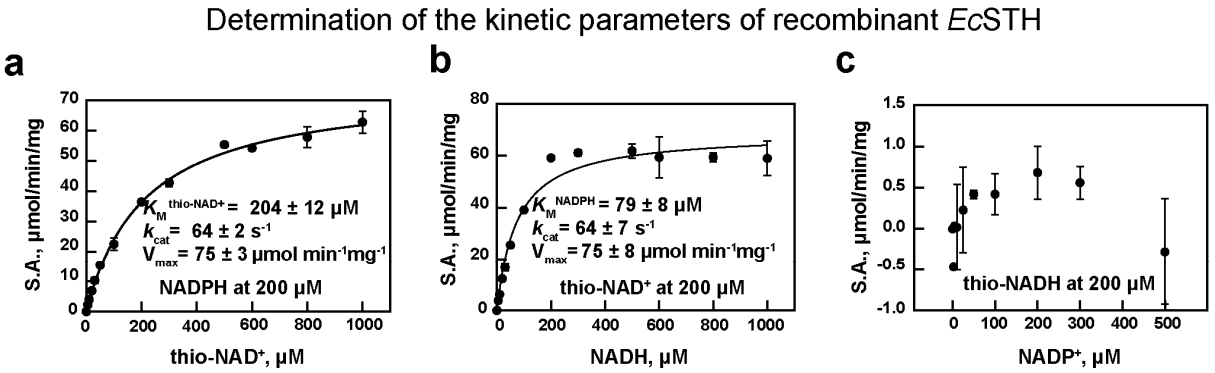


Supplementary Figure 1. Screening of bacterial STHs in HeLa cells. Effect of STHs expression from *E. coli*, *P. putida* and *A. vinelandii* on amounts of NAD^+ (**a**), NADH (**b**), total NAD pool (NAD^+ + NADH) (**c**), $NADP^+$ (**d**), NADPH (**e**) and total NADP pool ($NADP^+$ + NADPH) (**f**). The values in (**a**-**f**) correspond to ratios in (**Fig. 1c-d**). The total NADH/NAD^+ (**g**) and NADPH/$NADP^+$ (**h**) ratios measured in *wild-type* HeLa cells 3 hours after changing to fresh pyruvate-free $DMEM^{+dFBS}$ with 1 µM antimycin A (ANT), 1 µM piericidin A (Pier), 1 µM oligomycin A (Oligo A), or 1 mM pyruvate (PYR). Values are mean ± s.d.; n = 15, 15, 13, 13, 13, 13, 6, 6 in (**a**), n = 15, 15, 12, 12, 7, 12, 6, 6 in (**b**), n = 15, 15, 12, 12, 12, 12, 6, 6 in (**c**-**f**), n = 3 in (**g**-**h**) biologically independent samples. The statistical significance indicated for (**g**-**h**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



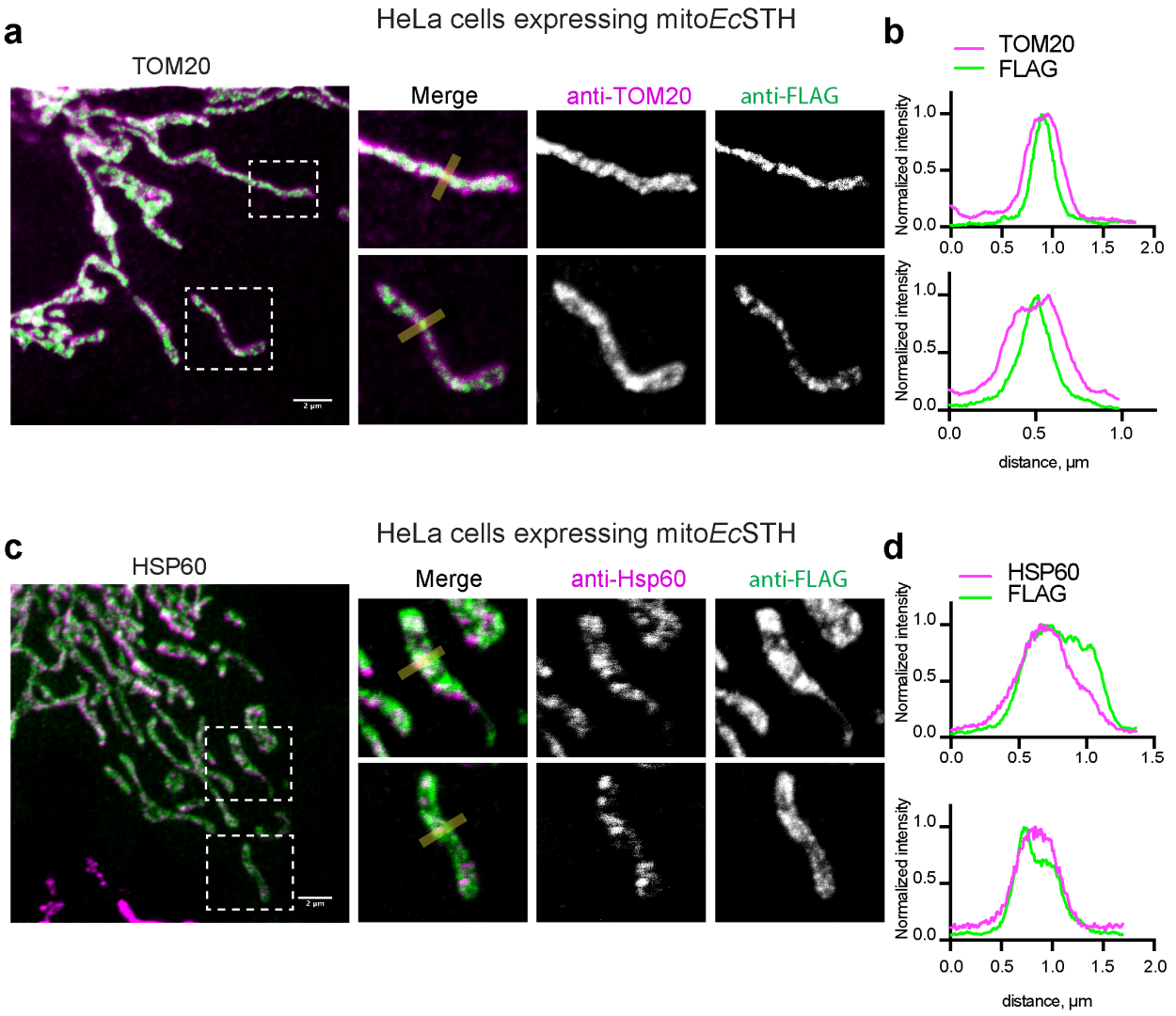
Supplementary Figure 2. Spent media lactate, pyruvate and proliferation of HeLa cells expressing bacterial STHs. Lactate (**a**) and pyruvate (**b**) levels in media which was incubated for 3 hours (spent media) with HeLa cells expressing untargeted and mitochondrially targeted STHs from *E. coli* and *P. putida*. The values in (**a**-**b**) correspond to ratios in (**Fig. 1e**). The effect of expression of untargeted and mitochondrially targeted STHs from *E. coli*, *P. putida* and *A. vinelandii* on proliferation of HeLa cells in pyruvate-free $DMEM^{+dFBS}$ with and without 300 ng/mL Dox (**c**). In (**d**) pyruvate-free $DMEM^{+dFBS}$ was supplemented with 1 µM antimycin A (ANT) and 200 µM uridine. Values are mean ± s.d.; n = 6 in (**a**-**b**) biologically independent samples. The statistical significance indicated for (**a**-**b**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference. For growth curves (**c**-**d**), error bars represent mean ± s.d.; n = 3 biologically independent samples.



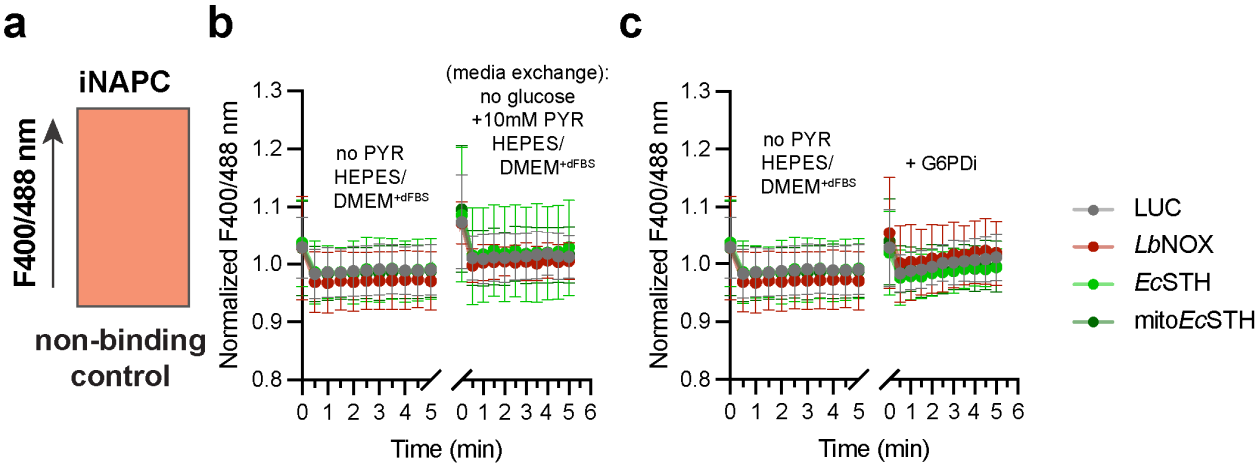
d Summary of the kinetic parameters of recombinant *Ec*STH

Substrate pair	Varying Substrate	K_m (µM)	V_{max} (µmol min^{-1} mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
thio-NAD$^+$ + NADPH	thio-NAD$^+$	204 ± 12	75 ± 3	64 ± 2	(3.2 ± 0.3) x 10^5
NAD$^+$ + thio-NADH	NAD$^+$	68 ± 8	12 ± 2	11 ± 2	(1.6 ± 0.1) x 10^5
NADPH + thio-NAD$^+$	NADPH	32 ± 3	35 ± 2	30 ± 1	(9.5 ± 0.7) x 10^5
NADH + thio-NAD$^+$	NADH	79 ± 8	75 ± 8	64 ± 7	(8.3 ± 0.3) x 10^5
NADP$^+$ + thio-NADH	NADP$^+$	*no reaction detected*			

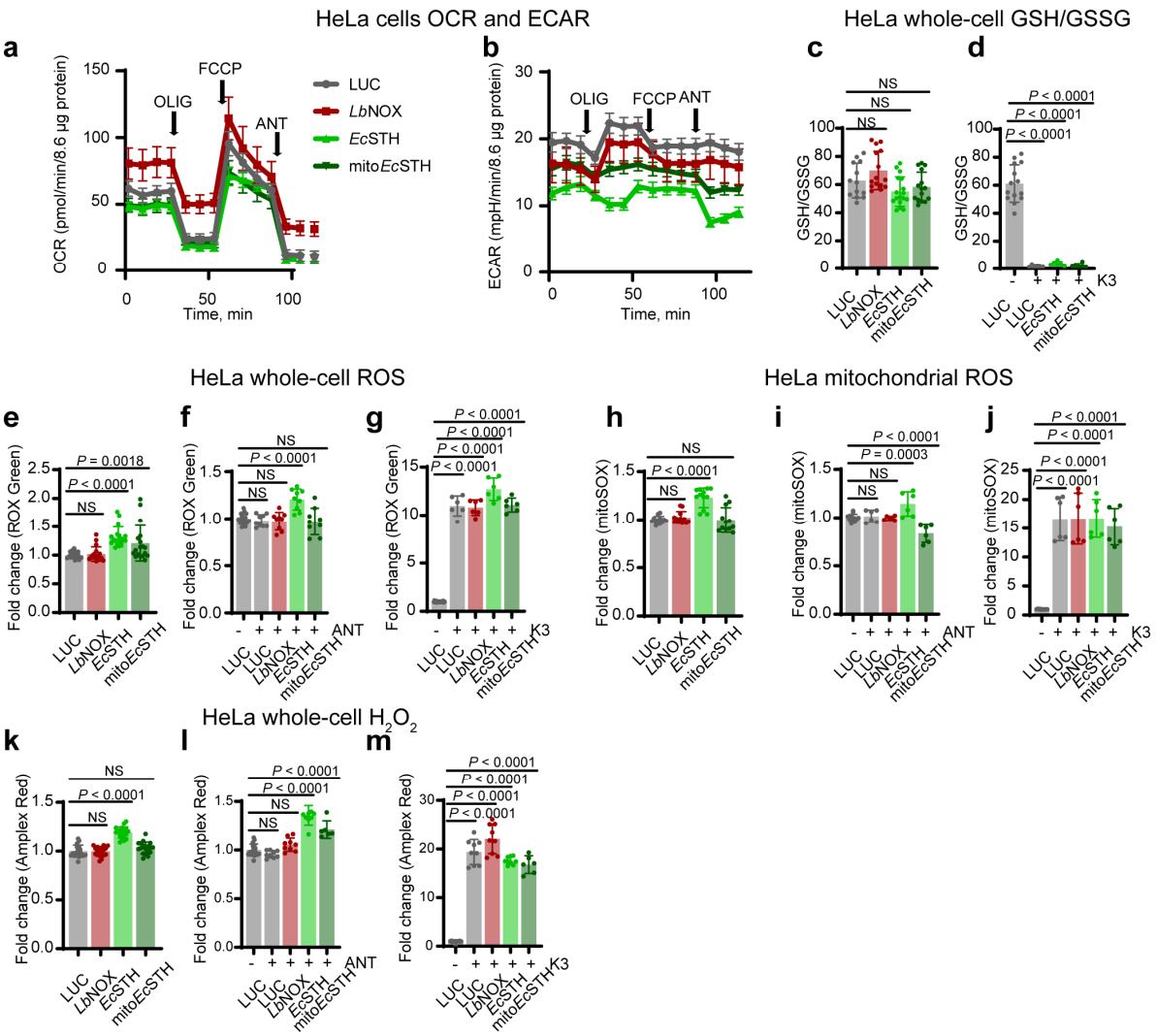
Supplementary Figure 3. Biochemical properties of recombinant *E. coli* STH (*Ec*STH). Michaelis-Menten analysis of the reaction catalyzed by *Ec*STH with thio-NAD$^+$ (**a**) or NADH (**b**). Fixed co-substrate at 200 µM was NADPH (**a**) and thio-NAD$^+$ (**b**). No reaction was observed for NADP$^+$ and thio-NADH (fixed at 200 µM) substrate pair (**c**). (**d**) Summary of the kinetic parameters for all substrate pairs tested in this study (see also **Fig. 2b-c**, Source Data file contains all values which were used to calculate kinetics parameters presented). Values are mean ± s.d.; n = 2 in (**a**-**c**) biologically independent samples. K_{cat} values were calculated per monomers of FAD active sites of *Ec*STH.



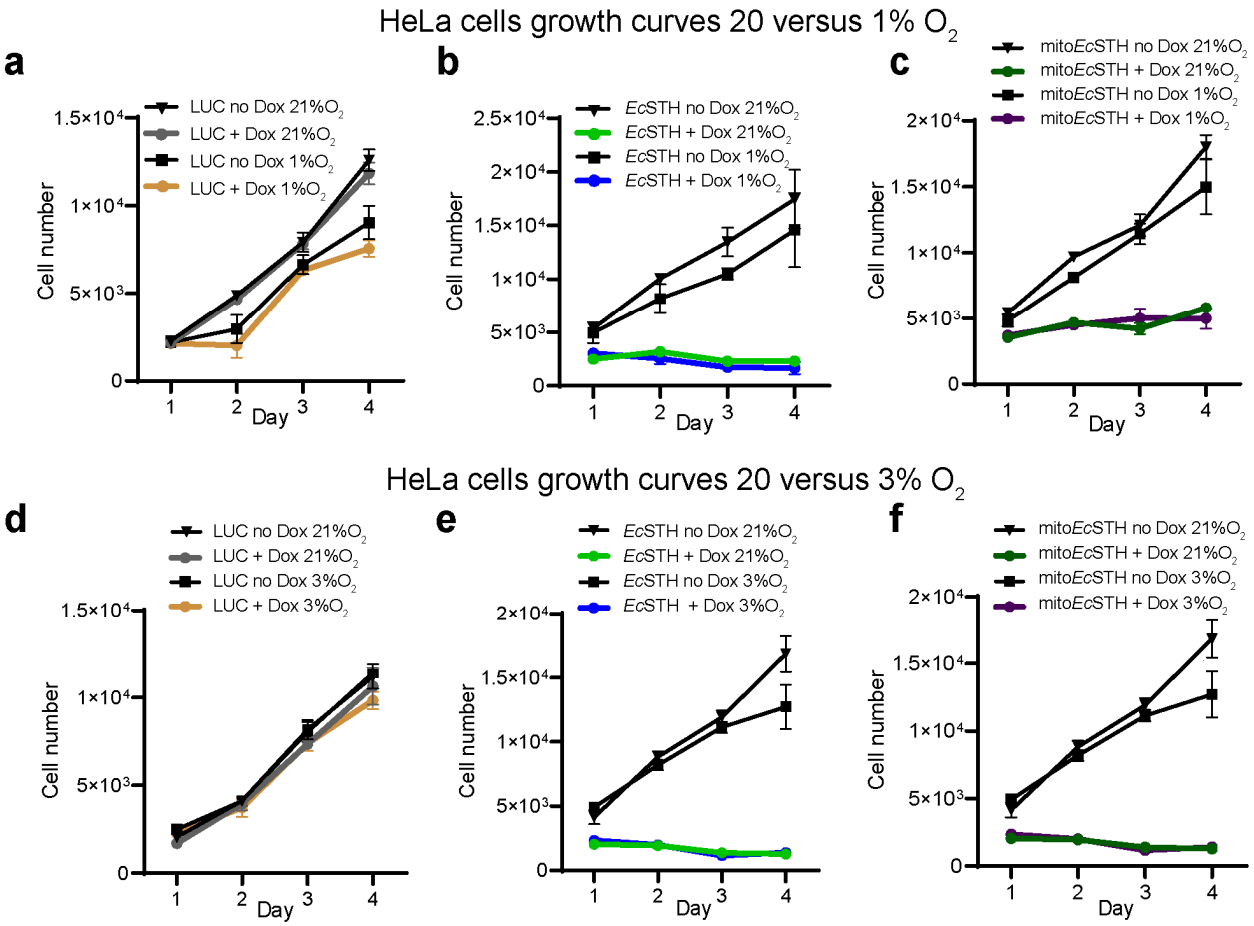
Supplementary Figure 4. Super resolution microscopy of HeLa cells expressing mito*Ec*STH. (**a**) Left panel is a representative super resolution image of HeLa cells expressing mito*Ec*STH (green, anti-FLAG; magenta, anti-TOM20 [mitochondria outer membrane marker]). Two sets of panels to the right are zoomed views of the two areas outlined by dashed boxes in the left panel. Individual channels of the zoomed areas are presented. (**b**) Line intensity profiles of cross-sections marked by light-yellow lines in (**a**) representing TOM20 (magenta) and FLAG (green) channels are shown. (**c**) Left panel is a representative super resolution image of HeLa cells expressing mito*Ec*STH (green, anti-FLAG; magenta, anti-HSP60 [mitochondria matrix marker]). Two sets of panels to the right are zoomed views of the two areas outlined by dash boxes in the left panel. Individual channels of the zoomed areas are presented. (**d**) Line intensity profiles of cross-sections marked by light-yellow lines in (**c**) representing HSP60 (magenta) and FLAG (green) channels are shown. Scale bars: 2 µm.



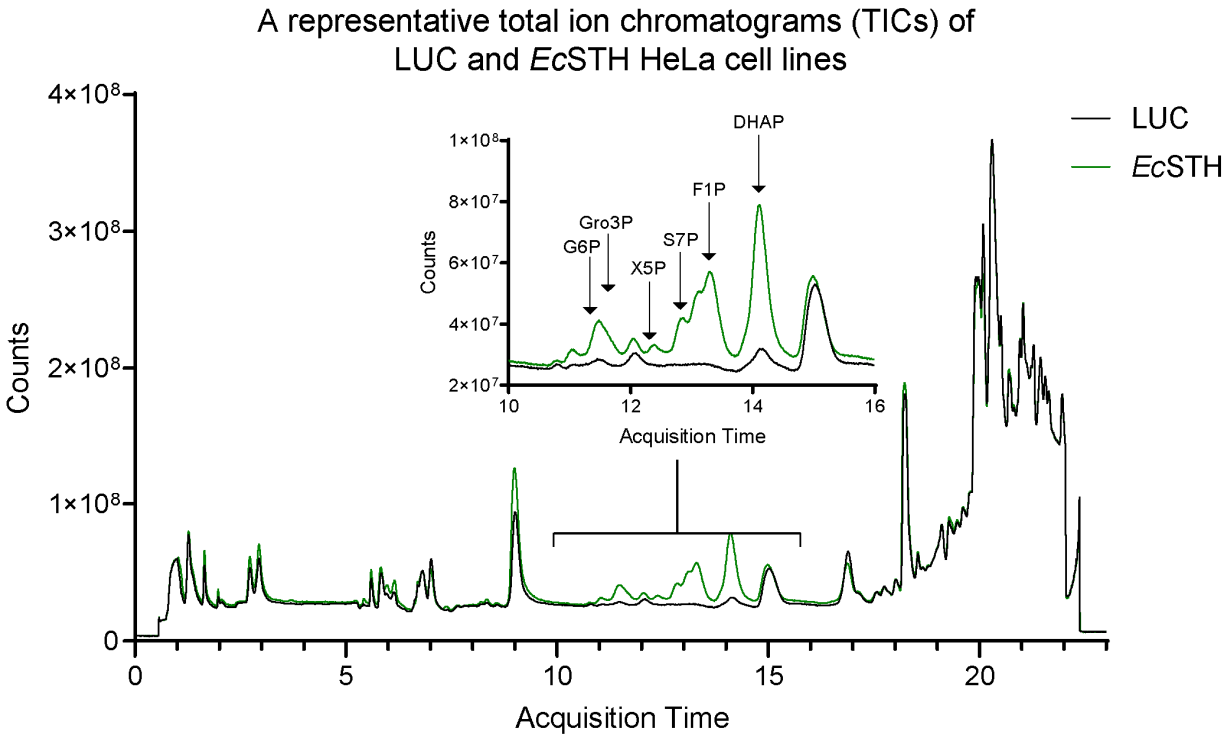
Supplementary Figure 5. Imaging of HeLa cells expressing *Ec*STH and mito*Ec*STH using inactive variant of the NADPH sensor. (**a**) iNAPC is a variant of iNAP that does not bind NADPH. Quantification of the time course measurements of the fluorescence ratio (F400/488) for HeLa cells with lentivirus mediated LUC, *Lb*NOX, *Ec*STH, mito*Ec*STH expression under Dox control transiently expressing iNAPC in the basal medium (DMEM without pyruvate, fluorescent vitamins and phenol red with 5 mM glucose, 25 mM HEPES, pH 7.4 and 1% dialyzed FBS) followed by replacement with basal medium without glucose but with 10 mM pyruvate (**b**) or when G6PDi was added (**c**). Values are mean ± s.d.; n = 9, 8, 9, 9 in (**b**), n = 9, 9, 9, 9 in (**c**) biologically independent samples.



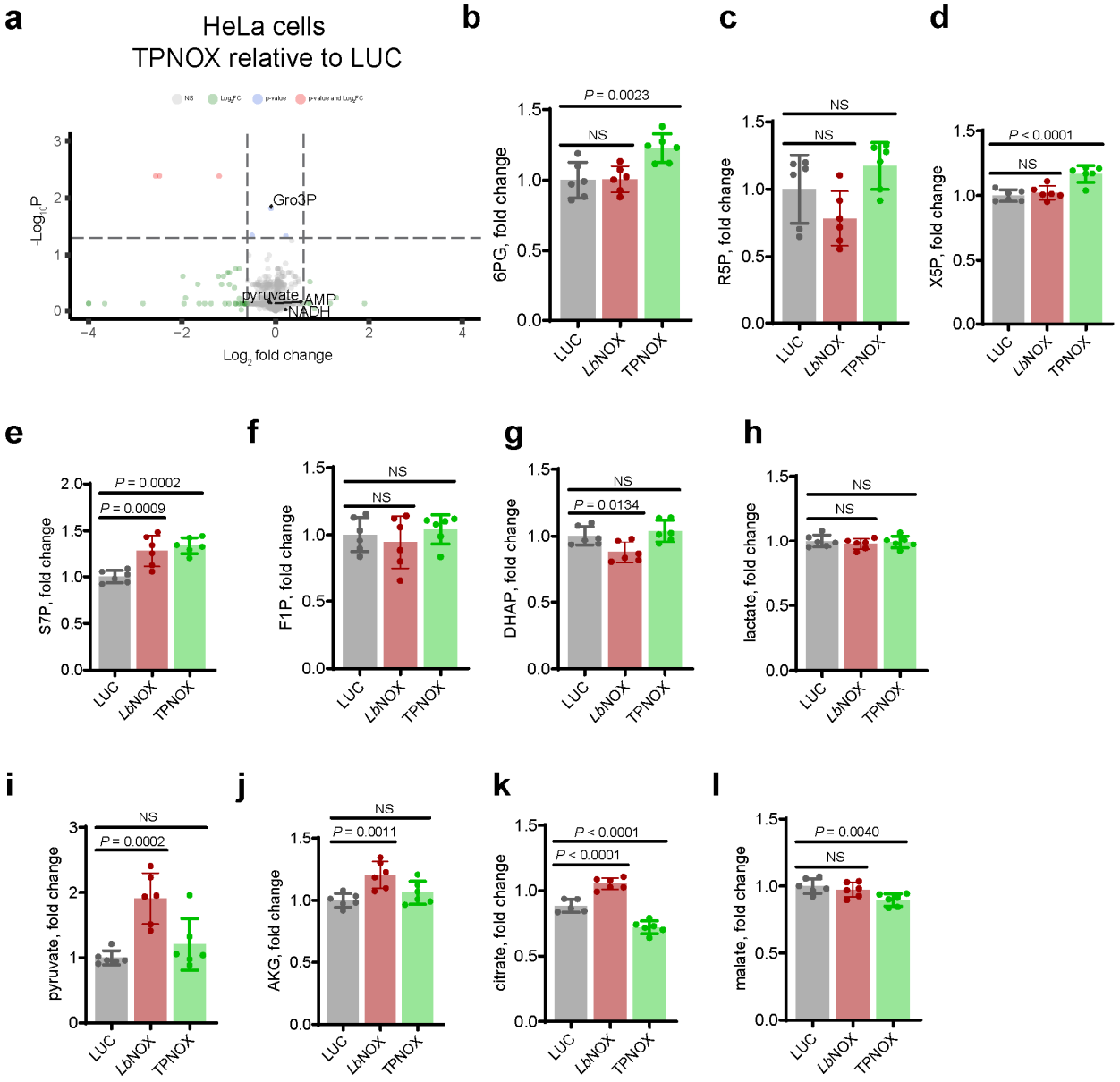
Supplementary Figure 6. Effect of *Ec*STH and mito*Ec*STH expression in HeLa cells on bioenergetics, GSH/GSSG ratios and reactive oxygen species (ROS) levels. (**a-b**) Mitochondrial stress test where OCR and ECAR were measured before and after sequential injection of 1 µM oligomycin A (OLIG), 2 µM FCCP, and 2 µM antimycin A (ANT). The GSH/GSSG ratios (**c-d**), ROS levels determined with CellROX Green (**e-g**), mitoSOX (**h-j**), and H_2O_2 levels determined with Amplex Red (**k-m**) in HeLa cells expressing *Ec*STH and mito*Ec*STH. Two hundred µM menadione (K3) or 1 µM antimycin A (ANT) were used as positive controls in (**d**, **f**, **g**, **i**, **j**, **l** and **m**). Values are mean ± s.d.; n = 12, 12, 11, 10 in (**a**), n = 12, 12, 11, 10 in (**b**), n = 13, 15, 17, 15 in (**c**), n = 14, 6, 6, 6 in (**d**), n = 18 in (**e**), n =18, 9, 9, 9, 9 in (**f**), n = 18, 6, 6, 6, 6 in (**g**), n = 12 in (**h**), n = 12, 6, 6, 6, 6 in (**i**), n = 12, 6, 6, 6, 6 in (**j**), n = 17, 17, 18, 18 in (**k**), n = 17, 9, 9, 9, 6 in (**l**), n = 17, 9, 9, 9, 6 in (**m**) biologically independent samples. The statistical significance indicated for (**c-m**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



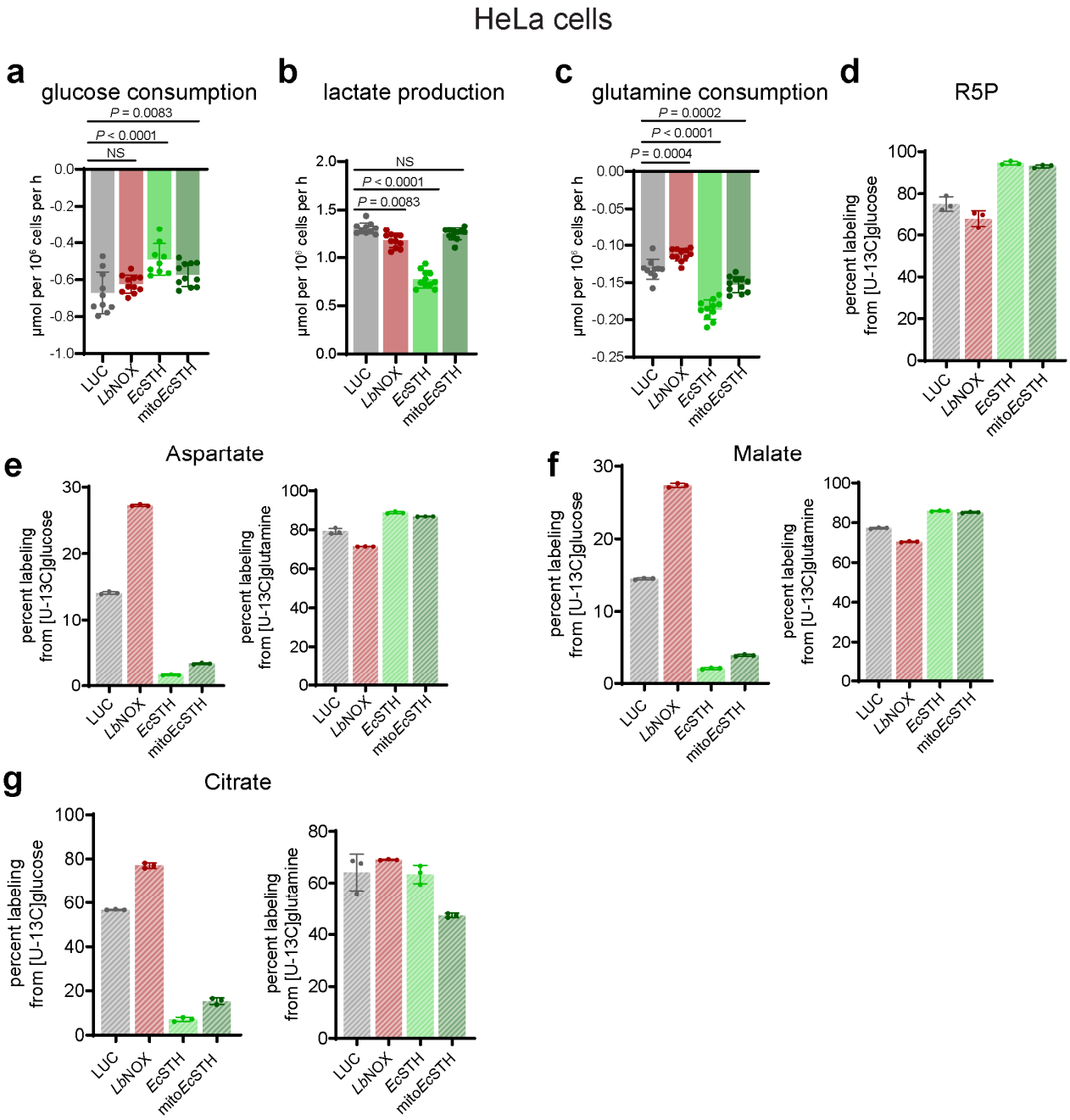
Supplementary Figure 7. Proliferation of HeLa cells expressing *Ec*STH and mito*Ec*STH in hypoxia. Proliferation of HeLa cells expressing LUC (**a**, **d**), *Ec*STH (**b**, **e**) and mito*Ec*STH (**c**, **f**) in pyruvate-free (-PYR) DMEM^{+dFBS} in 1%O_2 or 3%O_2 hypoxia. Proliferation of corresponding HeLa cell lines was determined with and without 300 ng/ml Dox. For growth curves (**a-f**) error bars represent mean ± s.d.; n = 6 biologically independent samples.



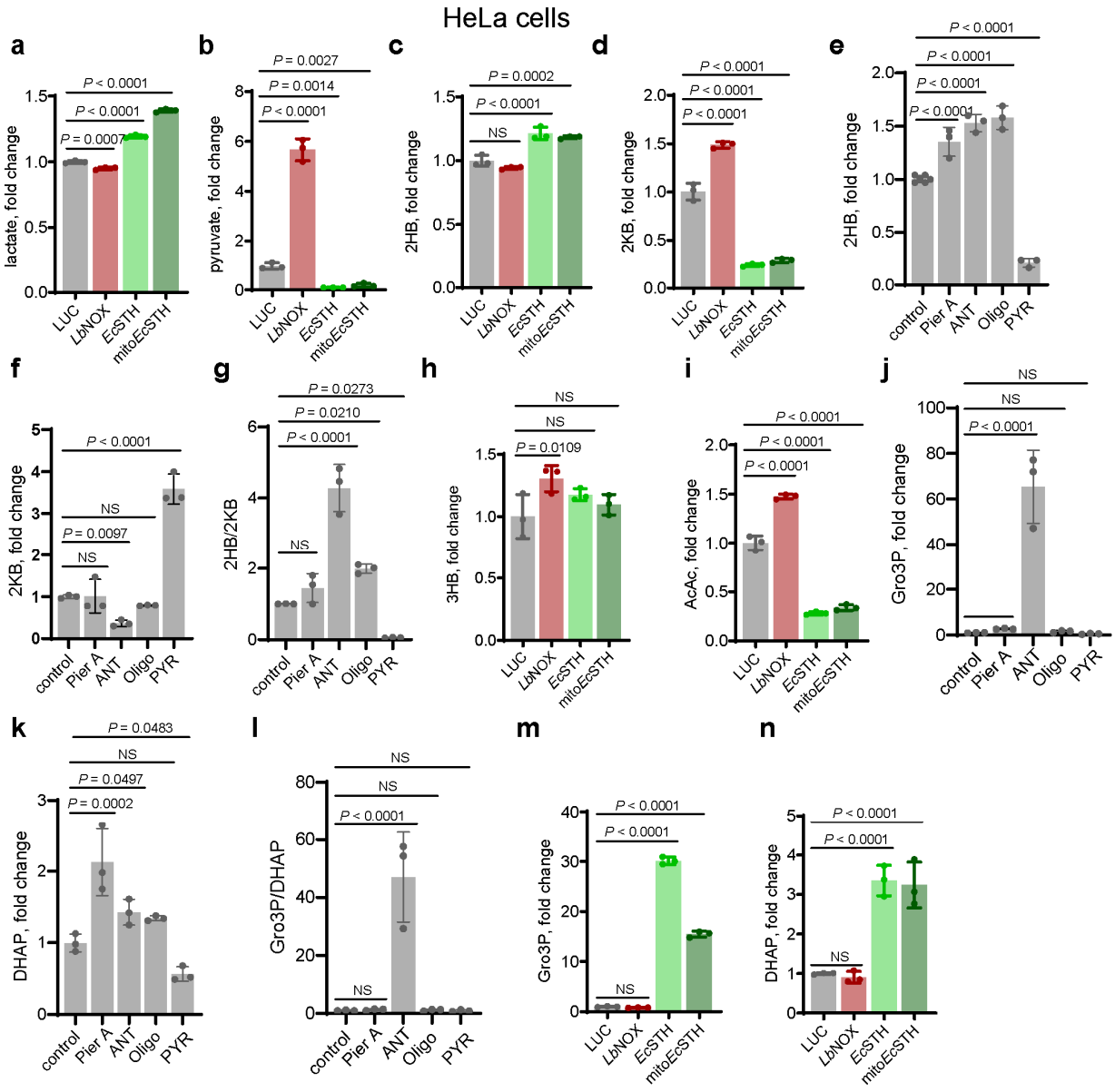
Supplementary Figure 8. NADH reductive stress leads to accumulation of non-canonical sugar phosphates. Total ion chromatograms (TICs) of HeLa cell lines expressing LUC or *Ec*STH. In HeLa cells *Ec*STH expression leads to increased signal intensity specifically in the chromatographic region where sugar phosphates and other glycolysis intermediates elute. Peaks which we were able to annotate are labeled. G6P: glucose-6-phosphate; Gro3P: glycerol 3-phosphate; X5P: xylulose-5-phosphate; S7P: sedoheptulose-7-phosphate; F1P: fructose-1-phosphate; DHAP: dihydroxyacetone phosphate.



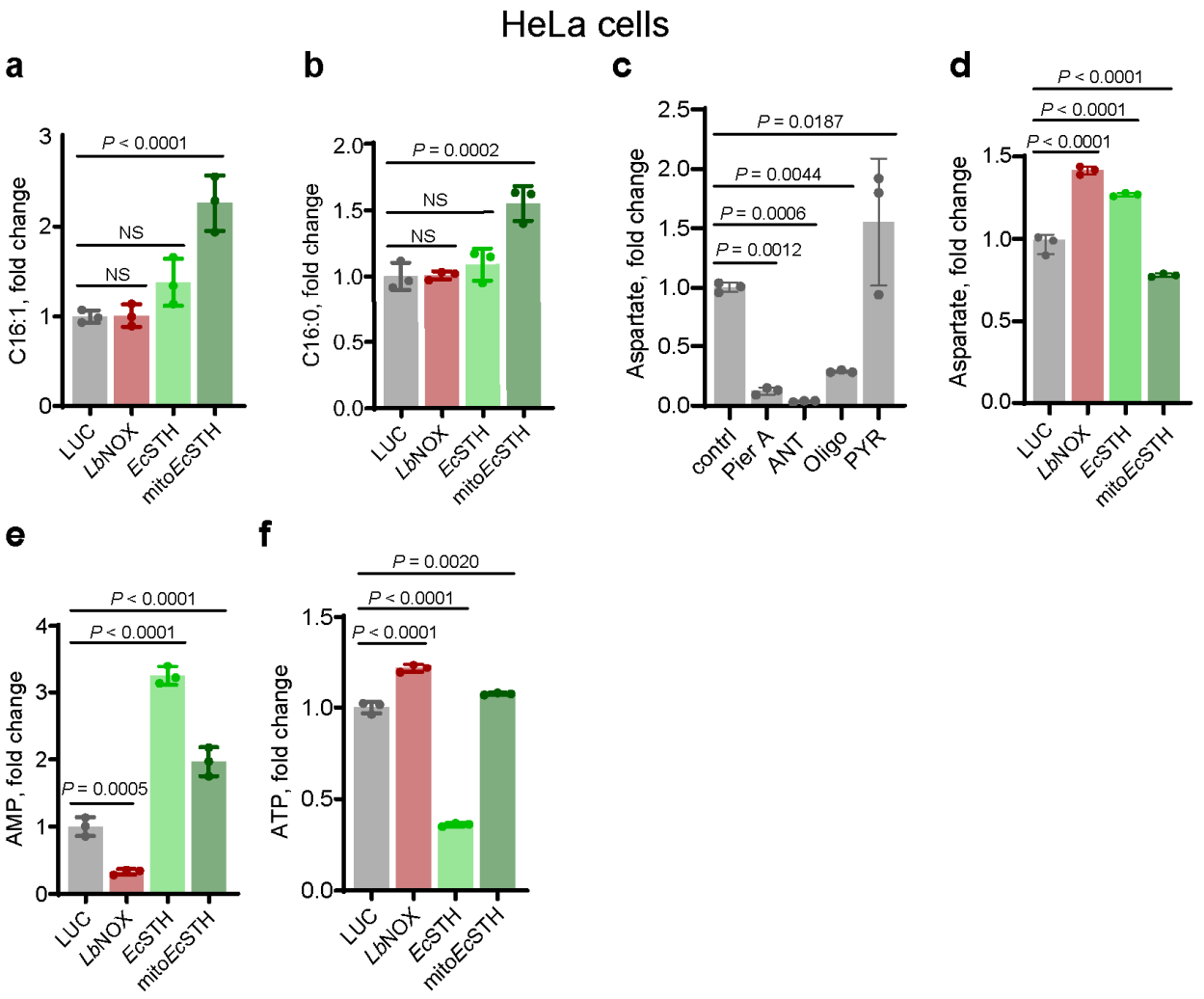
Supplementary Figure 9. Metabolic features of TPNOX expression in HeLa cells. (**a**) Untargeted metabolomics of HeLa cells expressing TPNOX relative to LUC control. The statistical significance indicated represents p value cutoff = 0.05, fold change cutoff = 1.5 and Welch t test (FDR corrected). Levels of 6PG (**b**), R5P (**c**), X5P (**d**), S7P (**e**), F1P (**f**), DHAP (**g**), lactate (**h**), pyruvate (**i**), AKG (**j**), citrate (**k**), and malate (**l**) in HeLa cells expressing TPNOX relative to LUC control. Values are mean ± s.d.; n = 6 in (**b-l**) biologically independent samples. The statistical significance indicated for (**b-l**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



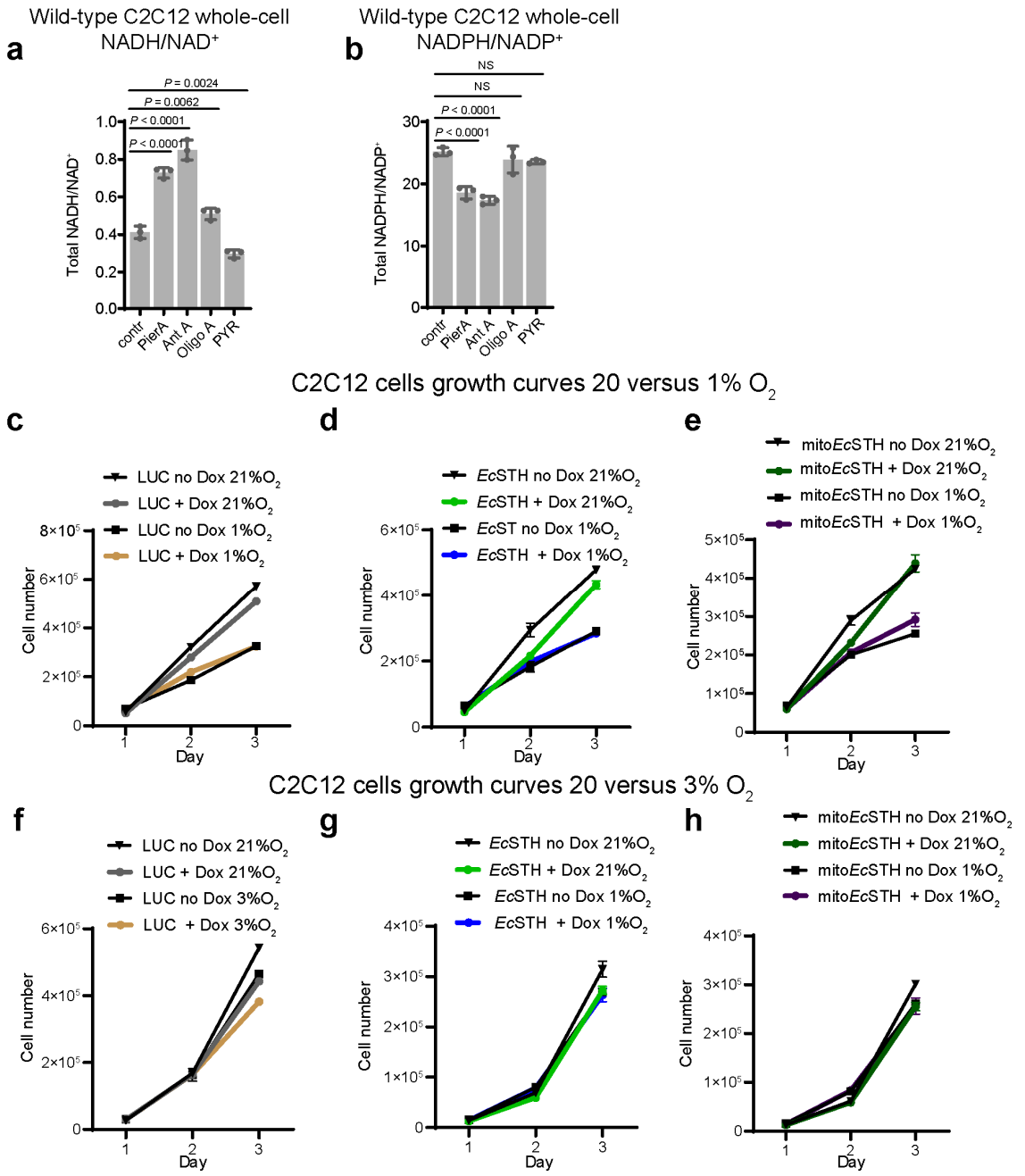
Supplementary Figure 10. Lactate production rates, glucose and glutamine consumption rates and stable isotope tracing analysis in HeLa cells expressing *Ec*STH and mito*Ec*STH. Glucose consumption (**a**), lactate production (**b**) and glutamine consumption (**c**) by HeLa expressing *Ec*STH and mito*Ec*STH. [U-$^{13}C_6$]glucose incorporation after 8 hours into ribose-5-phosphate (R5P) in HeLa cells with *Ec*STH and mito*Ec*STH expression (**d**). [U-$^{13}C_6$]glucose and [U-$^{13}C_5$]glutamine incorporation after 8 hours into aspartate (**e**), malate (**f**) and citrate (**g**) in HeLa cells with *Ec*STH and mito*Ec*STH expression. Values are mean ± s.d.; n = 10, 11, 10, 11 in (**a**), n = 10, 11, 11, 11 in (**b**), n = 10, 11, 11, 11 in (**c**), n = 3 in (**d-g**) biologically independent samples. The statistical significance indicated for (**a-c**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



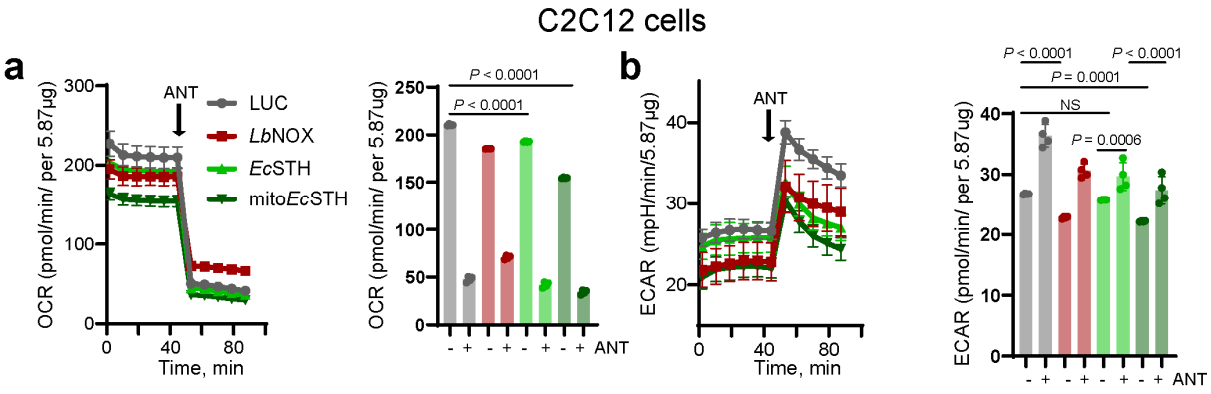
Supplementary Figure 11. Levels of various metabolites in HeLa cells expressing *Ec*STH and mito*Ec*STH. Lactate (**a**), pyruvate (**b**), 2HB (**c**), 2KB (**d**) levels in HeLa cells expressing *Ec*STH and mito*Ec*STH. 2HB (**e**), 2KB (**f**), 2HB/2KB ratio (**g**) in *wild-type* HeLa cells three hours after changing to fresh pyruvate-free DMEM^{+dFBS} with 1 µM piericidin A (Pier A), 1 µM antimycin A (ANT), 1 µM oligomycin A (Oligo A), or with 1 mM pyruvate (PYR). 3HB (**h**) and AcAc (**i**) levels in HeLa cells expressing *Ec*STH and mito*Ec*STH. Gro3P (**j**), DHAP (**k**), Gro3P/DHAP ratio (**l**) in *wild-type* HeLa cells three hours after changing to fresh pyruvate-free DMEM^{+dFBS} with 1 µM piericidin A (Pier A), 1 µM antimycin A (ANT), 1 µM oligomycin A (Oligo A), or with 1 mM pyruvate (PYR). Gro3P (**m**) and DHAP (**n**) levels in HeLa cells expressing *Ec*STH and mito*Ec*STH. Values are mean ± s.d.; n = 3 in (**a**-**n**) biologically independent samples. The statistical significance indicated for (**a**-**n**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



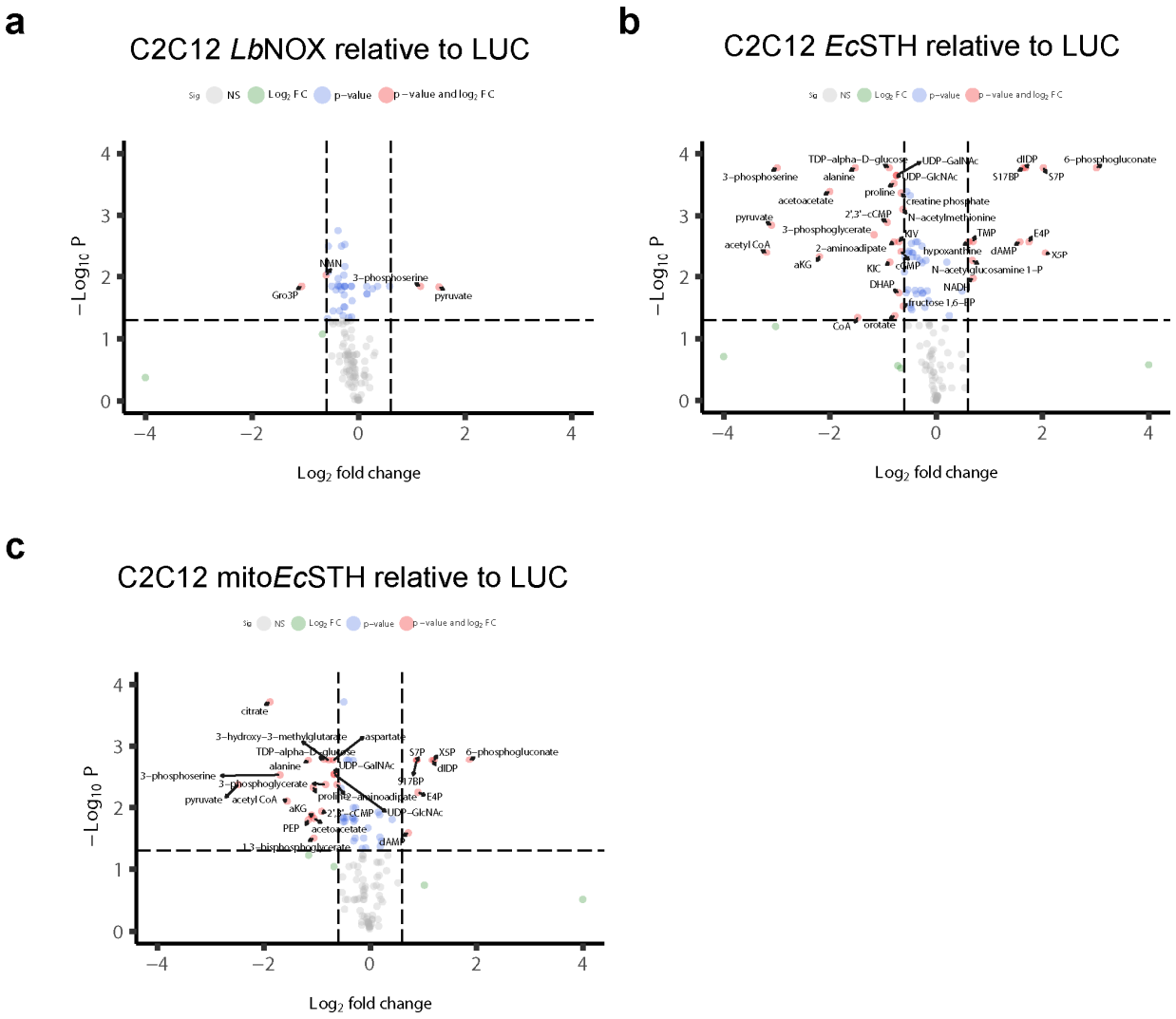
Supplementary Figure 12. Selected metabolites in HeLa cells expressing *Ec*STH and mito*Ec*STH. C16:1 (**a**), C16:0 (**b**) levels in HeLa cells expressing *Ec*STH and mito*Ec*STH. Aspartate (**c**) levels in *wild-type* HeLa cells three hours after changing to fresh pyruvate-free DMEM^{+dFBS} with 1 µM piericidin A (Pier A), 1 µM antimycin A (ANT), 1 µM oligomycin A (Oligo), or with 1 mM pyruvate (PYR). Aspartate (**d**), AMP (**e**) and ATP (**f**) levels in HeLa cells expressing *Ec*STH and mito*Ec*STH. Values are mean ± s.d.; n = 3 in (**a**-**f**) biologically independent samples. The statistical significance indicated for (**a**-**f**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



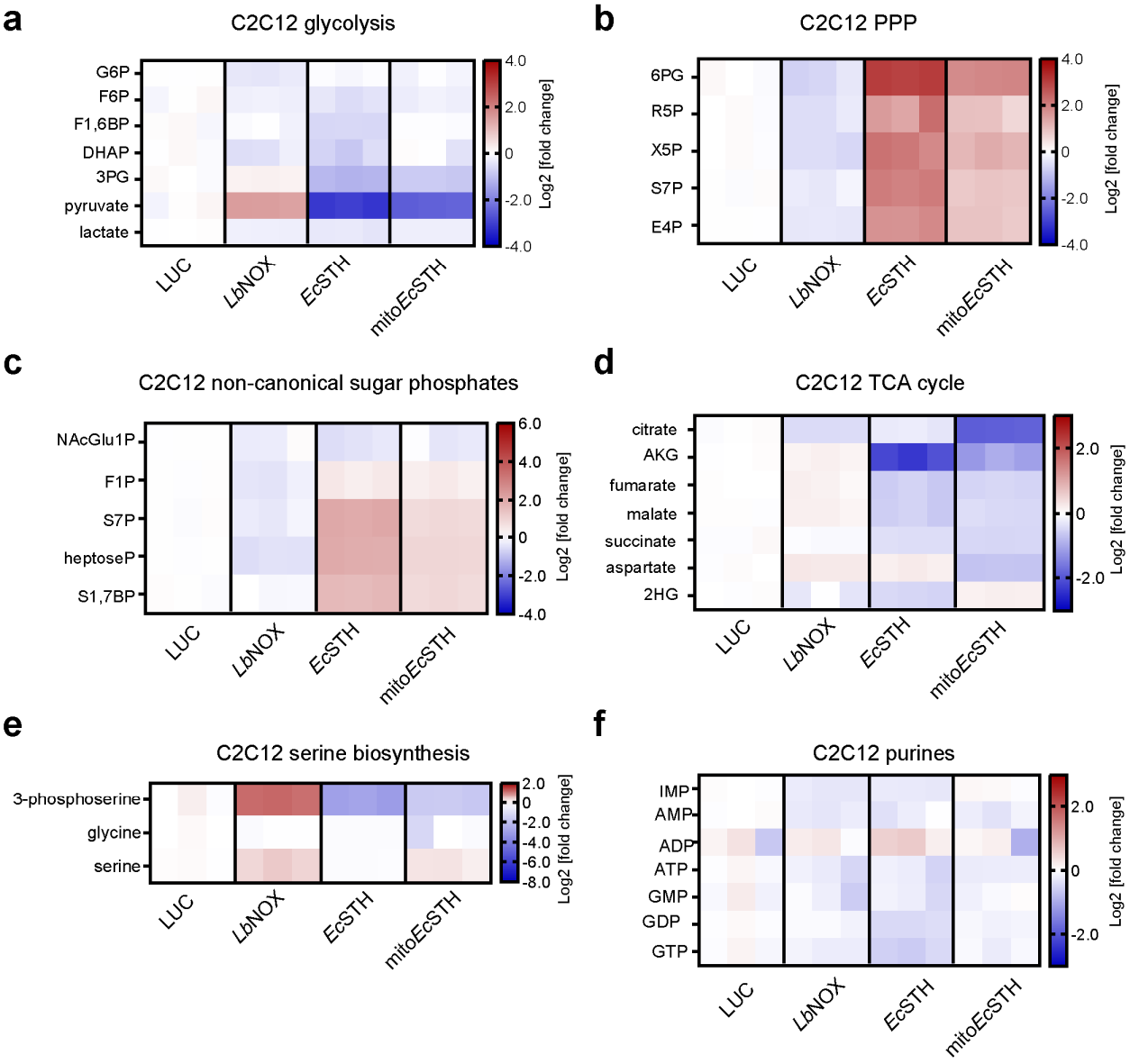
Supplementary Figure 13. Effect of ETC inhibition on NAD(P)H/NAD(P)$^+$ ratios in *wild-type* C2C12 cells and proliferation of C2C12 cells with *Ec*STH and mito*Ec*STH expression. The total NADH/NAD$^+$ (**a**) and NADPH/NADP$^+$ (**b**) ratios measured in *wild-type* C2C12 cells 3 hours after changing to fresh pyruvate-free DMEM^{+dFBS} with 1 μM antimycin A (ANT), 1 μM piericidin A (Pier), 1 μM oligomycin A (Oligo A), or 1 mM pyruvate (PYR). Proliferation of C2C12 cells expressing LUC (**c**, **f**), *Ec*STH (**d**, **g**) and mito*Ec*STH (**e**, **h**) in pyruvate-free (-PYR) DMEM^{+dFBS} in 1% O_2 or 3% O_2 hypoxia. Proliferation of corresponding C2C12 cell lines was determined with and without 300 ng/ml Dox. Values are mean ± s.d.; n = 3 (**a**-**b**) biologically independent samples. The statistical significance indicated for (**a**-**b**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference. For growth curves (**c**-**h**), error bars represent mean ± s.d.; n = 3 biologically independent samples.



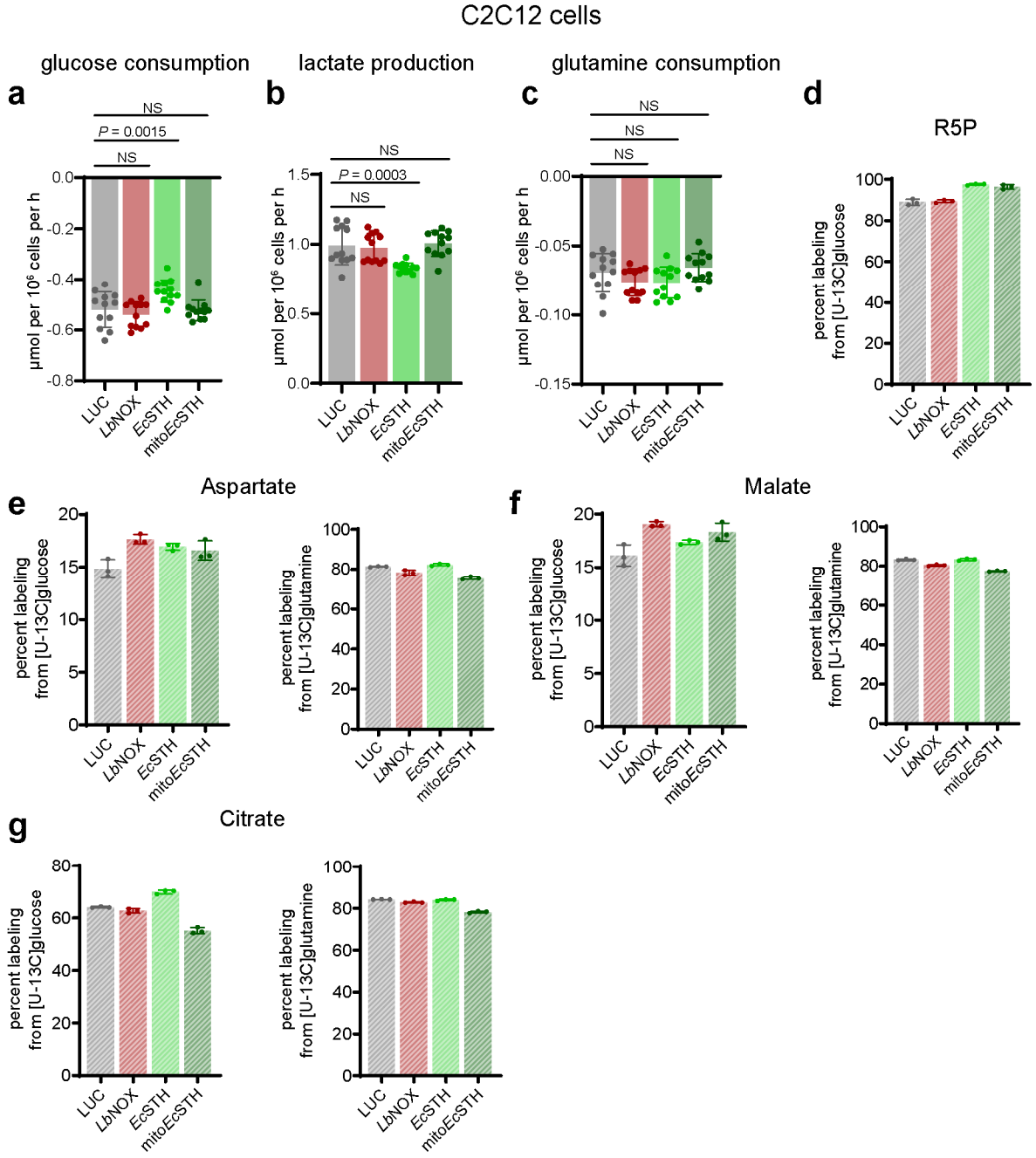
Supplementary Figure 14. Impact of *Ec*STH and mito*Ec*STH expression on oxygen consumption and acidification rate in C2C12 cells. Oxygen consumption rate (OCR) (**a**) and extracellular acidification rate (ECAR) (**b**) of C2C12 cells expressing *Ec*STH and mito*Ec*STH before and after addition of 1 µM antimycin A (ANT) measured in pyruvate free HEPES/DMEM^{+dFBS} media. Values are mean ± s.d.; n = 6, 6, 6, 5 for OCR traces in (**a**), n = 4 for bar graphs depicting OCR quantification in (**a**), n = 6, 6, 6, 5 for ECAR tracers in (**b**), n = 4 for bar graphs depicting ECAR quantification in (**b**) biologically independent samples. The statistical significance indicated for (**a**-**b**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.



Supplementary Figure 15. Targeted metabolomics of C2C12 cells expressing *Ec*STH and mito*Ec*STH. Targeted metabolomics in C2C12 cells expressing *Lb*NOX (**a**), *Ec*STH (**b**) and mito*Ec*STH (**c**). The statistical significance indicated for (**a-c**) represents p value cutoff = 0.05, fold change cutoff = 1.5 and Welch t test (FDR corrected).



Supplementary Figure 16. Metabolic features of *Ec*STH and mito*Ec*STH expression in C2C12 cells. Heatmaps of the most impacted glycolysis (**a**), pentose phosphate pathway (**b**), non-canonical sugar phosphates (**c**), the TCA cycle (**d**), serine biosynthesis (**e**) and purine biosynthesis (**f**) intermediates in C2C12 cells expressing *Ec*STH and mito*Ec*STH. G6P: glucose-6-phosphate; F6P: fructose-6-phosphate; F1,6BP: fructose-1,6-biphosphate; DHAP: dihydroxyacetone phosphate; 3PG: 3-phosphoglycerate; 6PG: 6-phosphogluconate; R5P: ribose-5-phosphate; X5P: xylulose-5-phosphate; S7P: sedoheptulose-7-phosphate, E4P: erythrose-4-phosphate; NAGlu1P: N-acetylglucosamine-1-phosphate; F1P: fructose-1-phosphate; heptoseP: a putative heptose with one phosphate; S1,7BP: sedoheptulose-1,7-bisphosphate; AKG: α-ketoglutarate; 2HG: 2-hydroxyglutarate. In heatmaps (**a**-**f**) each column represents biologically independent sample.



Supplementary Figure 17. Lactate production rates, glucose and glutamine consumption rates and stable isotope tracing analysis in C2C12 cells expressing *Ec*STH and mito*Ec*STH. Glucose consumption (**a**), lactate production (**b**) and glutamine consumption (**c**) by C2C12 cells expressing *Ec*STH and mito*Ec*STH. [U-$^{13}C_6$]glucose incorporation after 8 hours into ribose-5-phosphate (R5P) in C2C12 cells expressing *Ec*STH and mito*Ec*STH (**d**). [U-$^{13}C_6$]glucose and [U-$^{13}C_5$]glutamine incorporation after 8 hours into aspartate (**e**), malate (**f**) and citrate (**g**) by C2C12 cells expressing *Ec*STH and mito*Ec*STH. Values are mean ± s.d.; n = 12 in (**a-c**), n = 3 in (**d-g**) biologically independent samples. The statistical significance indicated for (**a-c**) represents a One-Way ANOVA followed by uncorrected Fisher's least significant difference test. NS, no significant difference.