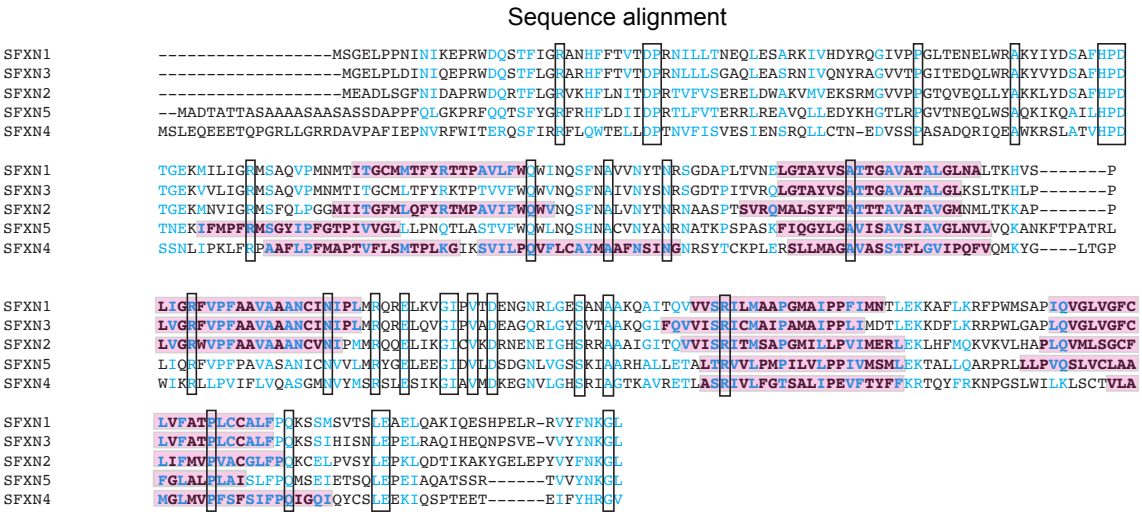
Cell Reports, Volume *34*

Supplemental information

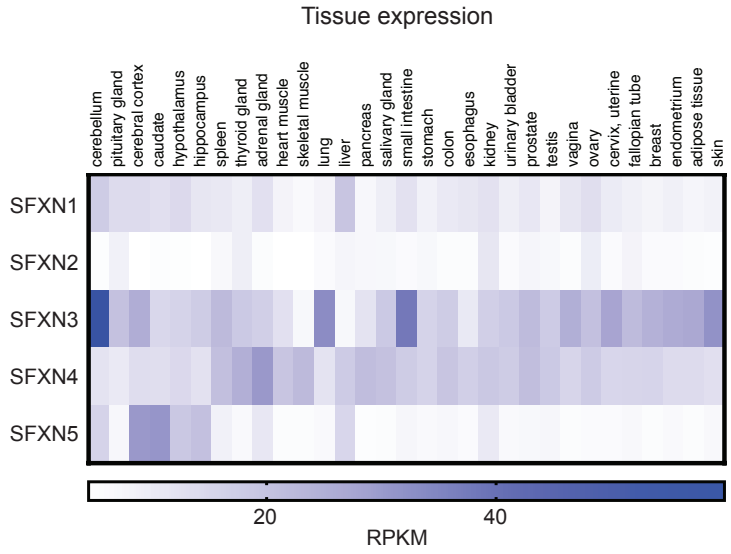
The mitochondrial carrier SFXN1 is critical for complex III integrity and cellular metabolism

Michelle Grace Acoba, Ebru S. Selen Alpergin, Santosh Renuse, Lucía Fernández-del-Río, Ya-Wen Lu, Oleh Khalimonchuk, Catherine F. Clarke, Akhilesh Pandey, Michael J. Wolfgang, and Steven M. Claypool

A



B



C

Figure S1

Figure S1, related to Figure 1. SFXN isoforms.
(A) Alignment of SFXN isoforms. Identical amino acids are boxed whereas highly similar ones are highlighted blue. Predicted transmembrane domains are in pink.
(B) Tissue distribution of SFXN isoforms. Gene expression is reported as median RPKM, generated by the GTEx project and accessed via the Human Protein Atlas.
(C) SFXN1 is highly conserved in eukaryotes. *Saccharomyces cerevisiae* possesses a related protein (Fsf1p).

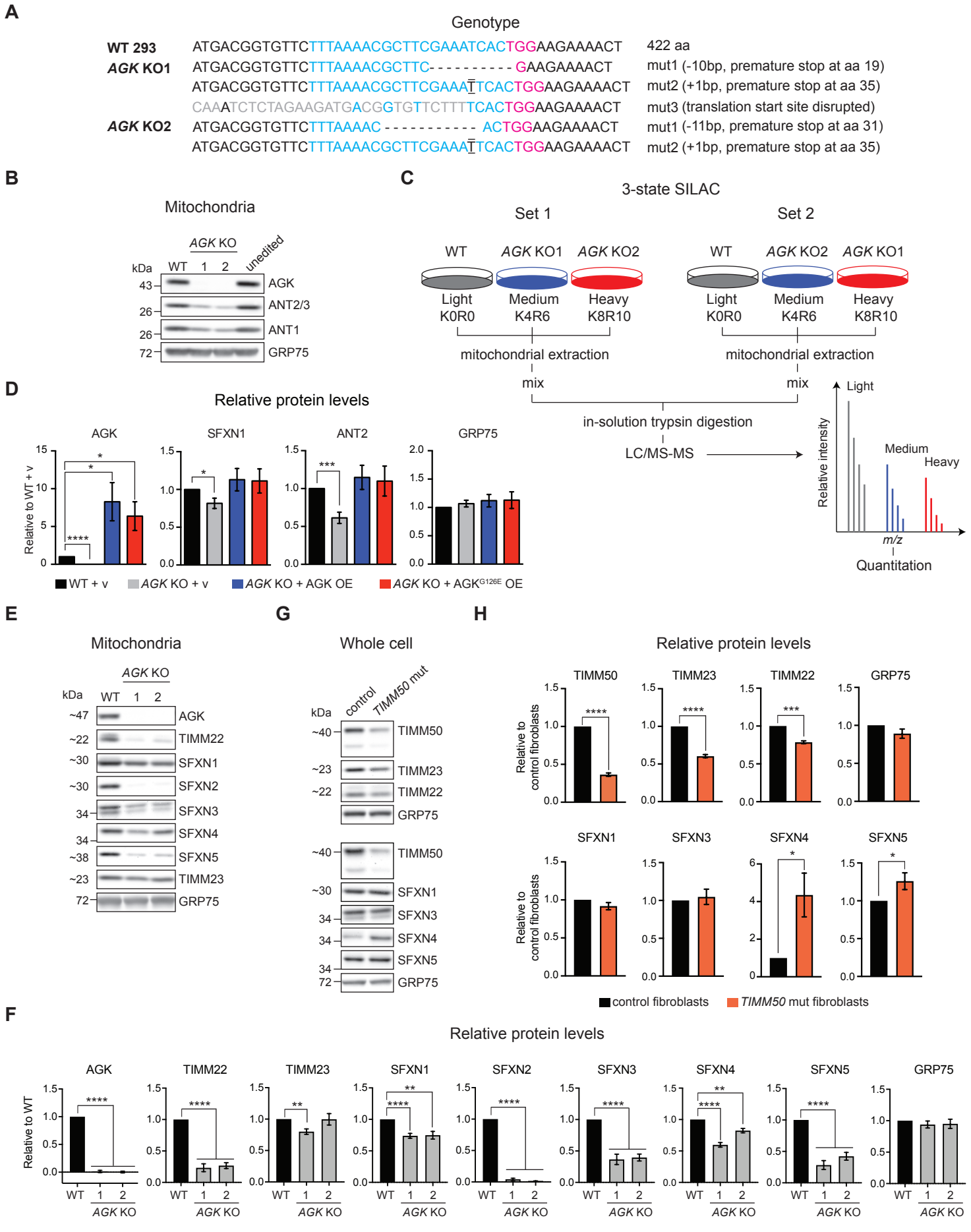

A
Genotype
WT 293
ATGACGGTGTTCTTTAAAACGCTTCGAAATCACTGGAAGAAAACT
422 aa
AGK KO1
ATGACGGTGTTCTTTAAAACGCTTC- - - - - - - - - - GAAGAAAACT
mut1 (-10bp, premature stop at aa 19)
ATGACGGTGTTCTTTAAAACGCTTCGAAATTCACTGGAAGAAAACT
mut2 (+1bp, premature stop at aa 35)
CAAATCTCTAGAAGATGACGGTGTTCTTTTCACTGGAAGAAAACT
mut3 (translation start site disrupted)
AGK KO2
ATGACGGTGTTCTTTAAAAC - - - - - - - - - - ACTGGAAGAAAACT
mut1 (-11bp, premature stop at aa 31)
ATGACGGTGTTCTTTAAAACGCTTCGAAATTCACTGGAAGAAAACT
mut2 (+1bp, premature stop at aa 35)
B
Mitochondria
AGK KO
kDa
WT
1
2
unedited
43
AGK
26
ANT2/3
26
ANT1
72
GRP75
C
3-state SILAC
Set 1
Set 2
WT
AGK KO1
AGK KO2
Light K0R0
Medium K4R6
Heavy K8R10
mitochondrial extraction
mix
in-solution trypsin digestion
LC/MS-MS
Light
Medium
Heavy
Relative intensity
m/z
Quantitation
D
Relative protein levels
AGK
SFXN1
ANT2
GRP75
Relative to WT + v
WT + v
AGK KO + v
AGK KO + AGK OE
AGK KO + AGK G126E OE
E
Mitochondria
AGK KO
kDa
WT
1
2
~47
AGK
~22
TIMM22
~30
SFXN1
~30
SFXN2
34
SFXN3
34
SFXN4
~38
SFXN5
~23
TIMM23
72
GRP75
G
Whole cell
control
TIMM50 mut
kDa
~40
TIMM50
~23
TIMM23
~22
TIMM22
GRP75
~40
TIMM50
~30
SFXN1
34
SFXN3
34
SFXN4
34
SFXN5
72
GRP75
H
Relative protein levels
TIMM50
TIMM23
TIMM22
GRP75
SFXN1
SFXN3
SFXN4
SFXN5
Relative to control fibroblasts
control fibroblasts
TIMM50 mut fibroblasts
F
Relative protein levels
AGK
TIMM22
TIMM23
SFXN1
SFXN2
SFXN3
SFXN4
SFXN5
GRP75
Relative to WT
WT
1
2
AGK KO

Figure S2

Figure S2, related to Figure 1. AGK disruption reduces steady-state protein levels of mitochondrial carriers including SFXNs.
(A) Genomic mutations documented in two HEK293-based *AGK* KOs generated by CRISPR/Cas9. gRNA target site is in blue, while the protospacer-adjacent motif (PAM) sequence is in red.
(B) Immunoblotting using mitochondrial isolates for AGK, ANT1, and ANT2/3 in WT, AGK-deficient clones, and an unedited clone that went through the transfection process with the CRISPR construct. GRP75 served as a loading control.
(C) SILAC scheme related to Figure 1J.
(D) Densitometric analysis of bands for proteins in Figure 1K. Protein levels in WT were set to 1.0. (mean±SEM, $n \geq 4$).
(E) Immunoblotting for different SFXN isoforms and select TIM subunits using mitochondrial isolates from WT and AGK-null clones. GRP75 served as a loading control.
(F) Densitometric analysis of bands for proteins in (E). Protein levels in WT were set to 1.0. (mean±SEM, $n \geq 4$).
(G) Immunoblotting for different SFXN isoforms and select TIM subunits using cell extracts from control and *TIMM50* mutation-containing patient fibroblasts. GRP75 served as a loading control. (H) Densitometric analysis of bands for proteins in (G). Protein levels in WT were set to 1.0. (mean±SEM, $n \geq 3$).
**p<0.05*, ***p<0.01*, ****p<0.001*, *****p<0.0001*, unpaired Student's t-test

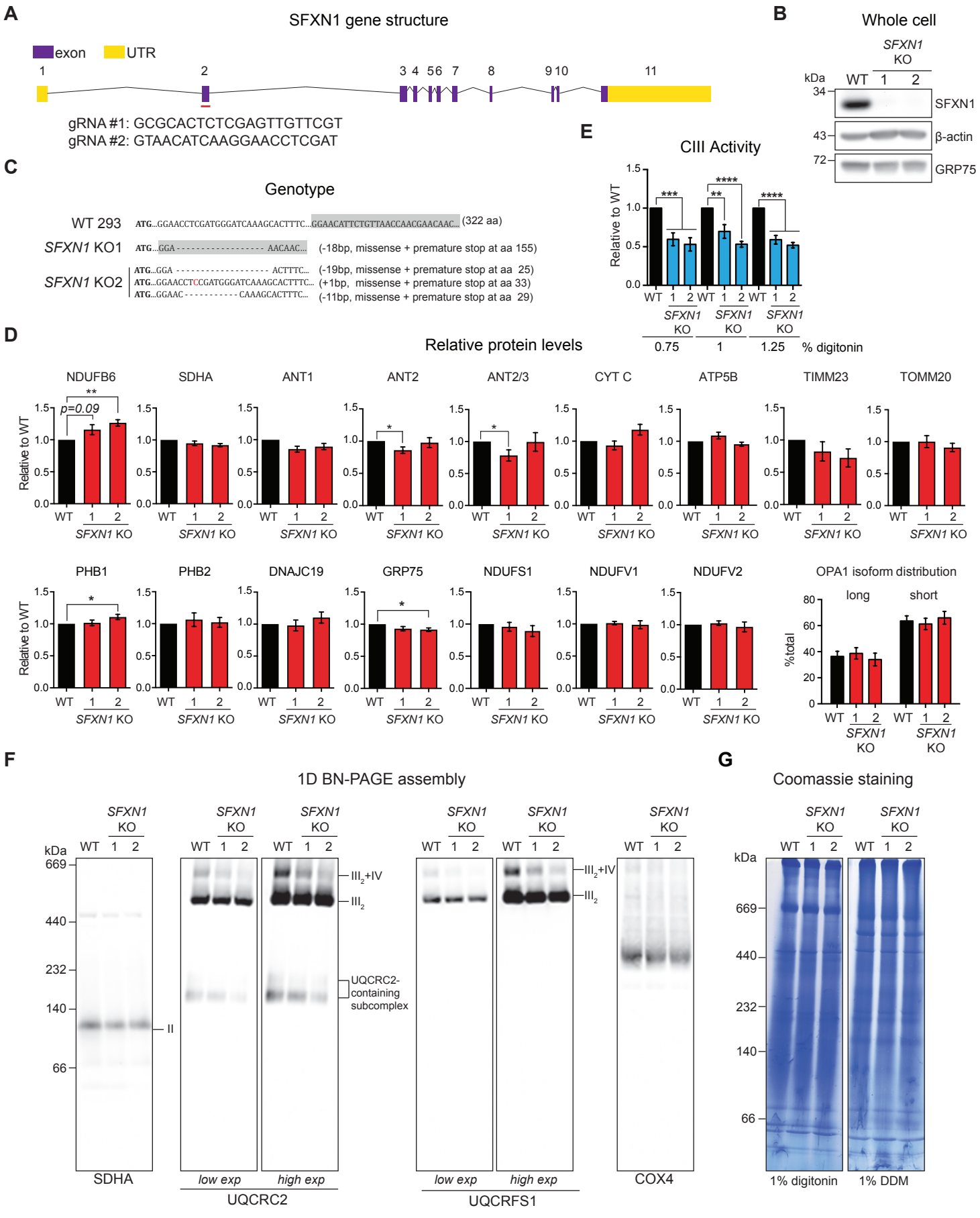

A
SFXN1 gene structure
exon
UTR
1
2
3 4 5 6 7
8
9 10
11
gRNA #1: GCGCACTCTCGAGTTGTTCGT
gRNA #2: GTAACATCAAGGAACCTCGAT
B
Whole cell
SFXN1 KO
kDa
WT 1 2
34
SFXN1
43
β-actin
72
GRP75
C
Genotype
WT 293
ATG...GGAACCTCGATGGGATCAAAGCACTTTC...GGAACATTCTGTTAACCAACGAACAAC... (322 aa)
SFXN1 KO1
ATG...GGA - - - - - - - - - - - - - - - - - AACAAC... (-18bp, missense + premature stop at aa 155)
SFXN1 KO2
ATG...GGA - - - - - - - - - - - - - - - - - ACTTTC... (-19bp, missense + premature stop at aa 25)
ATG...GGAACCTCCGATGGGATCAAAGCACTTTC... (+1bp, missense + premature stop at aa 33)
ATG...GGAAC - - - - - - - - - - CAAAGCACTTTC... (-11bp, missense + premature stop at aa 29)
E
CIII Activity
Relative to WT
1.5
1.0
0.5
0.0
***
**
****
****
WT 1 2 WT 1 2 WT 1 2
SFXN1 KO
SFXN1 KO
SFXN1 KO
0.75
1
1.25
% digitonin
D
Relative protein levels
NDUFB6
p=0.09
SDHA
ANT1
ANT2
ANT2/3
CYT C
ATP5B
TIMM23
TOMM20
PHB1
PHB2
DNAJC19
GRP75
NDUFS1
NDUFV1
NDUFV2
Relative to WT
WT 1 2
SFXN1 KO
OPA1 isoform distribution
long
short
%total
80
60
40
20
0
F
1D BN-PAGE assembly
SFXN1 KO
kDa
669
440
232
140
66
II
III2+IV
III2
UQCRC2-containing subcomplex
SDHA
low exp
high exp
UQCRC2
UQCRFS1
COX4
G
Coomassie staining
1% digitonin
1% DDM

Figure S3

Figure S3, related to Figures 2 and 3. Effects of SFXN1 ablation on the levels, function and assembly of respiratory complexes.

(A) *SFXN1* gene structure showing the target sites of gRNAs used for CRISPR/Cas9.

(B) Whole cell extracts from WT and *SFXN1* KO clones were analyzed by SDS-PAGE and immunoblotting.

(C) Genotyping shows genetic lesions resulting in premature stop codons in both clones.

(D) Densitometric analysis of bands for other proteins immunoblotted as shown in Figure 2A. Protein levels in WT were set to 1.0. (mean±SEM, $n \geq 4$).

(E) Complex III activity was measured by solubilizing mitochondria with increasing concentrations of digitonin and monitoring cytochrome *c* reduction at 550 nm (mean±SEM, $n=6$).

(F) 1D BN assembly of respiratory complexes I-IV was assessed by solubilizing mitochondria in 1% (w/v) DDM, resolving in a 4-16% BN gel, and immunoblotting for the indicated complex subunits.

(G) Coomassie staining of mitochondria solubilized with 1% (w/v) digitonin or 1% (w/v) DDM and resolved on 6-16% BN gel.

*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, unpaired Student's t-test

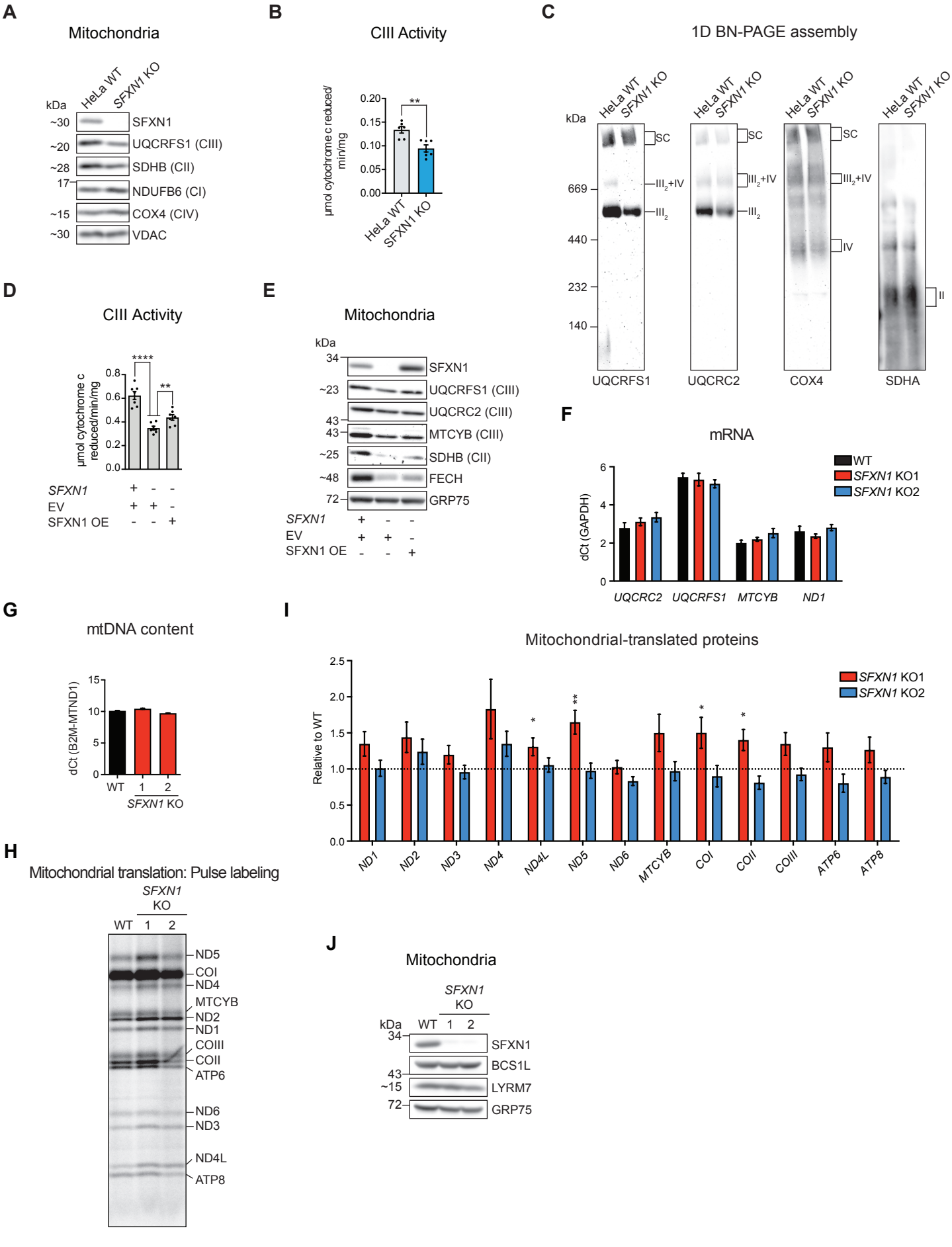

A
Mitochondria
HeLa WT
SFXN1 KO
kDa
~30
SFXN1
~20
UQCRFS1 (CIII)
~28
SDHB (CII)
17
NDUFB6 (CI)
~15
COX4 (CIV)
~30
VDAC
B
CIII Activity
µmol cytochrome c reduced/ min/mg
0.20
0.15
0.10
0.05
0.00
**
HeLa WT
SFXN1 KO
C
1D BN-PAGE assembly
HeLa WT
SFXN1 KO
kDa
669
440
232
140
SC
III2+IV
III2
IV
II
UQCRFS1
UQCRC2
COX4
SDHA
D
CIII Activity
µmol cytochrome c reduced/min/mg
0.8
0.6
0.4
0.2
0.0
****
**
SFXN1
+ - -
EV
+ + -
SFXN1 OE
- - +
E
Mitochondria
kDa
34
SFXN1
~23
UQCRFS1 (CIII)
UQCRC2 (CIII)
43
43
MTCYB (CIII)
~25
SDHB (CII)
~48
FECH
72
GRP75
SFXN1
+ - -
EV
+ + -
SFXN1 OE
- - +
F
mRNA
WT
SFXN1 KO1
SFXN1 KO2
dCt (GAPDH)
6
4
2
0
UQCRC2
UQCRFS1
MTCYB
ND1
G
mtDNA content
dCt (B2M-MTND1)
15
10
5
0
WT
1
2
SFXN1 KO
I
Mitochondrial-translated proteins
SFXN1 KO1
SFXN1 KO2
Relative to WT
2.5
2.0
1.5
1.0
0.5
0.0
*
**
*
*
ND1
ND2
ND3
ND4
ND4L
ND5
ND6
MTCYB
COI
COII
COIII
ATP6
ATP8
H
Mitochondrial translation: Pulse labeling
SFXN1 KO
WT
1
2
ND5
COI
ND4
MTCYB
ND2
ND1
COIII
COII
ATP6
ND6
ND3
ND4L
ATP8
J
Mitochondria
SFXN1 KO
kDa
WT
1
2
34
SFXN1
BCS1L
43
~15
LYRM7
72
GRP75

Figure S4

Figure S4, related to Figure 3. Complex III-associated function of SFXN1 does not derive from changes in mtDNA content, its transcription or translation, or altered CIII assembly factor levels.
HeLa was used for (A) to (C). HEK293 was used for the remainder of the experiments.
(A) Immunoblotting for select OXPHOS subunits using mitochondrial extracts.
(B) Complex III activity was measured after solubilizing mitochondria with 0.5% (w/v) DDM and monitoring cytochrome *c* reduction at 550 nm (mean±SEM, *n*=6).
(C) 1D BN assembly of Complexes II-IV was analyzed by solubilizing mitochondria in 1% (w/v) digitonin, resolving clarified extracts on a 4-16% BN gel, and immunoblotting for the indicated subunits.
(D) Complex III activity was measured after solubilizing mitochondria with 0.5% (w/v) DDM and monitoring cytochrome *c* reduction at 550 nm (mean±SEM, *n*=7).
(E) Immunoblotting for select mitochondrial proteins using mitochondrial isolates.
(F) Gene expression analysis of CIII subunits *UQCRC2*, *UQCRFS1* and *MTCYB* by quantitative PCR (qPCR). *ND1*, a Complex I subunit, served as a control. Data presented as mean fold-change (FC)±SEM (*n*=6) relative to HEK293 WT. FC was calculated using the $2^{-\Delta\Delta CT}$ method. GAPDH served as the housekeeping gene.
(G) mtDNA content determination by qPCR. Genomic DNA isolated from the indicated cell lines served as template. mtDNA levels are presented as mean dC_T±SEM (*n*=6) between the mitochondria-encoded *ND1* and the nuclear-encoded reference gene β-2-microglobulin (*β2M*).
(H) Mitochondrial translation experiments. Cytosolic translation was inhibited by anisomycin (100 µg/mL), and mitochondrially-encoded polypeptides were pulse-labeled for 1 hr with ^{35}S-methionine/cysteine (200 µCi/mL). Bands were detected by phosphoimaging.
(I) Densitometric band analysis was performed and the distribution of signals from mitochondrial-translated products was calculated as a percentage of the total signal per experiment (mean % total signal±SEM, *n=6*).
(J) Immunoblotting for CIII assembly factors using mitochondrial extracts.
**p<0.05*, ***p<0.01*, ****p<0.001*, *****p<0.0001,* unpaired Student's t-test

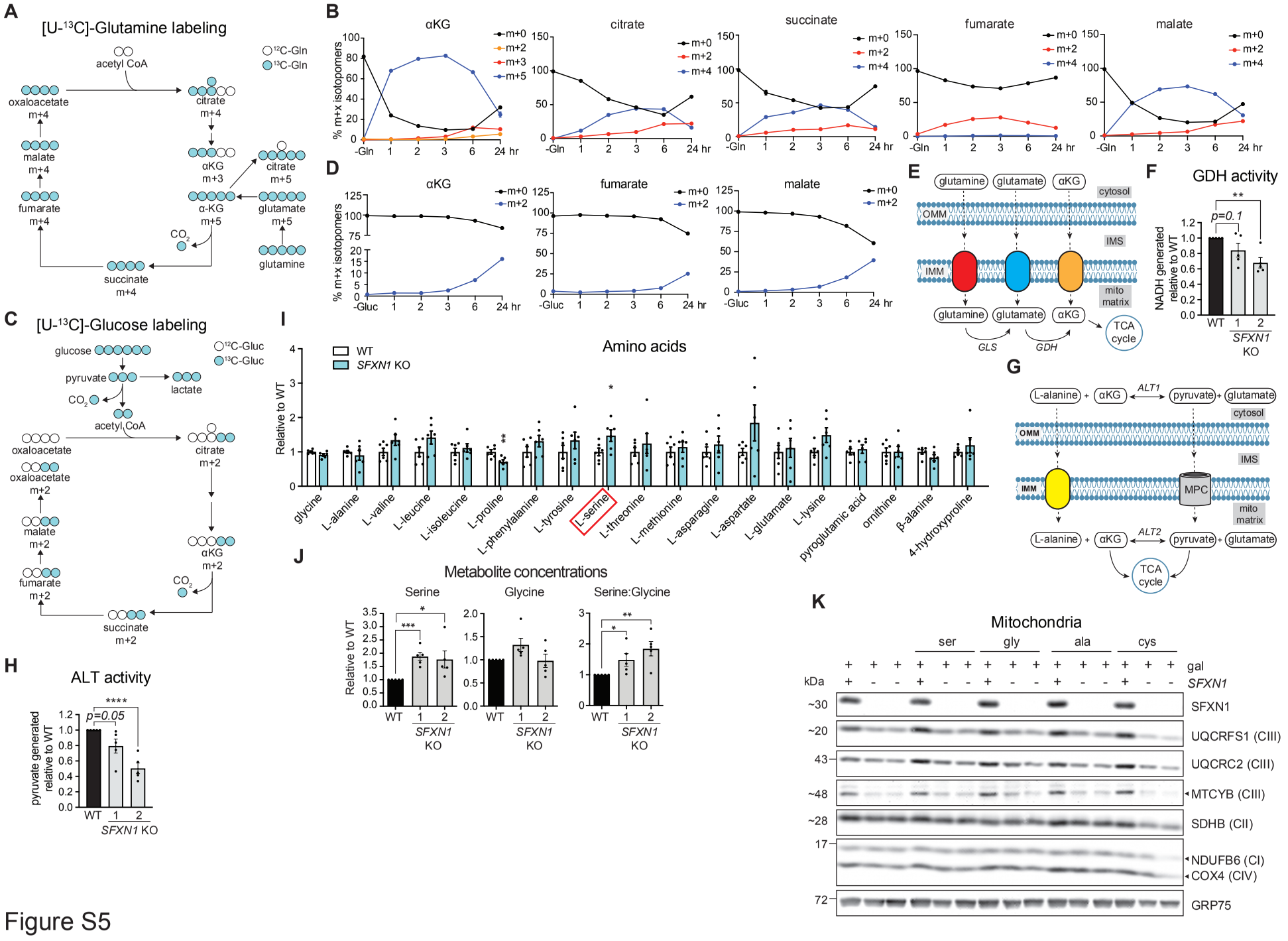

A
[U-13C]-Glutamine labeling
12C-Gln
13C-Gln
acetyl CoA
oxaloacetate m+4
citrate m+4
malate m+4
αKG m+3
citrate m+5
fumarate m+4
α-KG m+5
glutamate m+5
CO2
glutamine
succinate m+4
B
αKG
citrate
succinate
fumarate
malate
% m+x isotopomers
m+0
m+2
m+3
m+4
m+5
-Gln
24 hr
C
[U-13C]-Glucose labeling
12C-Gluc
13C-Gluc
glucose
pyruvate
lactate
CO2
acetyl CoA
oxaloacetate m+2
citrate m+2
malate m+2
αKG m+2
fumarate m+2
succinate m+2
D
αKG
fumarate
malate
-Gluc
E
glutamine
glutamate
αKG
cytosol
OMM
IMS
IMM
mito matrix
TCA cycle
GLS
GDH
F
GDH activity
p=0.1
NADH generated relative to WT
WT
SFXN1 KO
G
L-alanine
ALT1
pyruvate
glutamate
ALT2
MPC
H
ALT activity
p=0.05
pyruvate generated relative to WT
I
Amino acids
WT
SFXN1 KO
Relative to WT
glycine
L-alanine
L-valine
L-leucine
L-isoleucine
L-proline
L-phenylalanine
L-tyrosine
L-serine
L-threonine
L-methionine
L-asparagine
L-aspartate
L-glutamate
L-lysine
pyroglutamic acid
ornithine
β-alanine
4-hydroxyproline
J
Metabolite concentrations
Serine
Glycine
Serine:Glycine
K
Mitochondria
ser
gly
ala
cys
gal
kDa
SFXN1
~30
~20
43
~48
~28
17
72
UQCRFS1 (CIII)
UQCRC2 (CIII)
MTCYB (CIII)
SDHB (CII)
NDUFB6 (CI)
COX4 (CIV)
GRP75

Figure S5

Figure S5, related to Figures 4, 6 and 7. Assessment of central carbon and amino acid metabolism in *SFXN1* KOs.
(A) Schematic diagram of [U-^{13}C]-glutamine labeling of TCA intermediates.
(B) Time-course experiment for [U-^{13}C]-glutamine labeling.
(C) Schematic diagram of [U-^{13}C]-glucose labeling of TCA intermediates.
(D) Time-course experiment for [U-^{13}C]-glucose labeling.
(E) Reaction mediated by glutamine dehydrogenase (GDH).
(F) GDH activity in cellular extracts of HEK293 WT and *SFXN1* KOs.
(G) Reaction mediated by alanine aminotransferase (ALT).
(H) ALT activity in cellular extracts of HEK293 WT and *SFXN1* KOs.
(I) Relative quantification of amino acids by GC-MS (mean±SEM, $n=6$), related to Figure 4C.
(J) Serine and glycine abundance in cellular extracts relative to WT as determined by fluorimetric-based assays. Serine:glycine ratio is also presented (mean±SEM, $n=5$).
(K) Immunoblotting for select respiratory complex subunits using mitochondrial isolates from cells grown for 2 days in galactose-based media only or supplemented with 2 mM serine, glycine, alanine or cystine.
*$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, unpaired Student's t-test

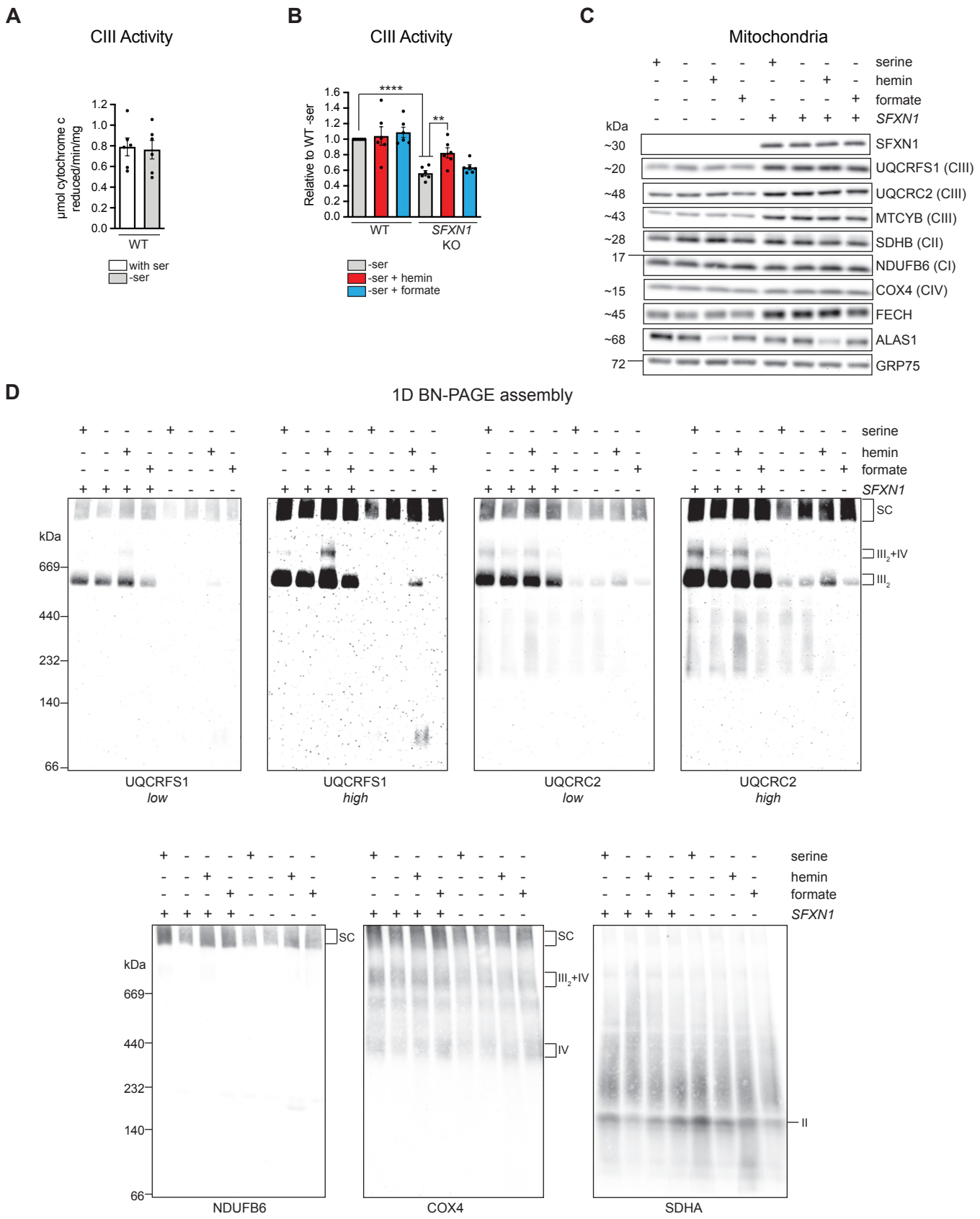

A
CIII Activity
µmol cytochrome c reduced/min/mg
WT
with ser
-ser
B
CIII Activity
Relative to WT -ser
****
**
WT
SFXN1 KO
-ser
-ser + hemin
-ser + formate
C
Mitochondria
serine
hemin
formate
SFXN1
kDa
SFXN1
UQCRFS1 (CIII)
UQCRC2 (CIII)
MTCYB (CIII)
SDHB (CII)
NDUFB6 (CI)
COX4 (CIV)
FECH
ALAS1
GRP75
D
1D BN-PAGE assembly
kDa
669
440
232
140
66
SC
III2+IV
III2
IV
II
UQCRFS1 low
UQCRFS1 high
UQCRC2 low
UQCRC2 high
NDUFB6
COX4
SDHA

Figure S6

Figure S6, related to Figure 7. Complex III function in the absence of serine and upon hemin and formate supplementation.
(A) Complex III activity in DDM-solubilized mitochondria from cells grown in glucose-containing media with or without serine (mean±SEM, *n*=6).
Mitochondrial isolates from cells grown in glucose-containing media without serine, or with addition of hemin or formate were used to assess:
(B) Complex III activity (mean±SEM, *n*=6),
(C) Steady-state abundance of select proteins, and
(D) 1D BN assembly of respiratory complexes.
**p<0.05*, ***p<0.01*, ****p<0.001*, *****p<0.0001,* unpaired Student's t-test

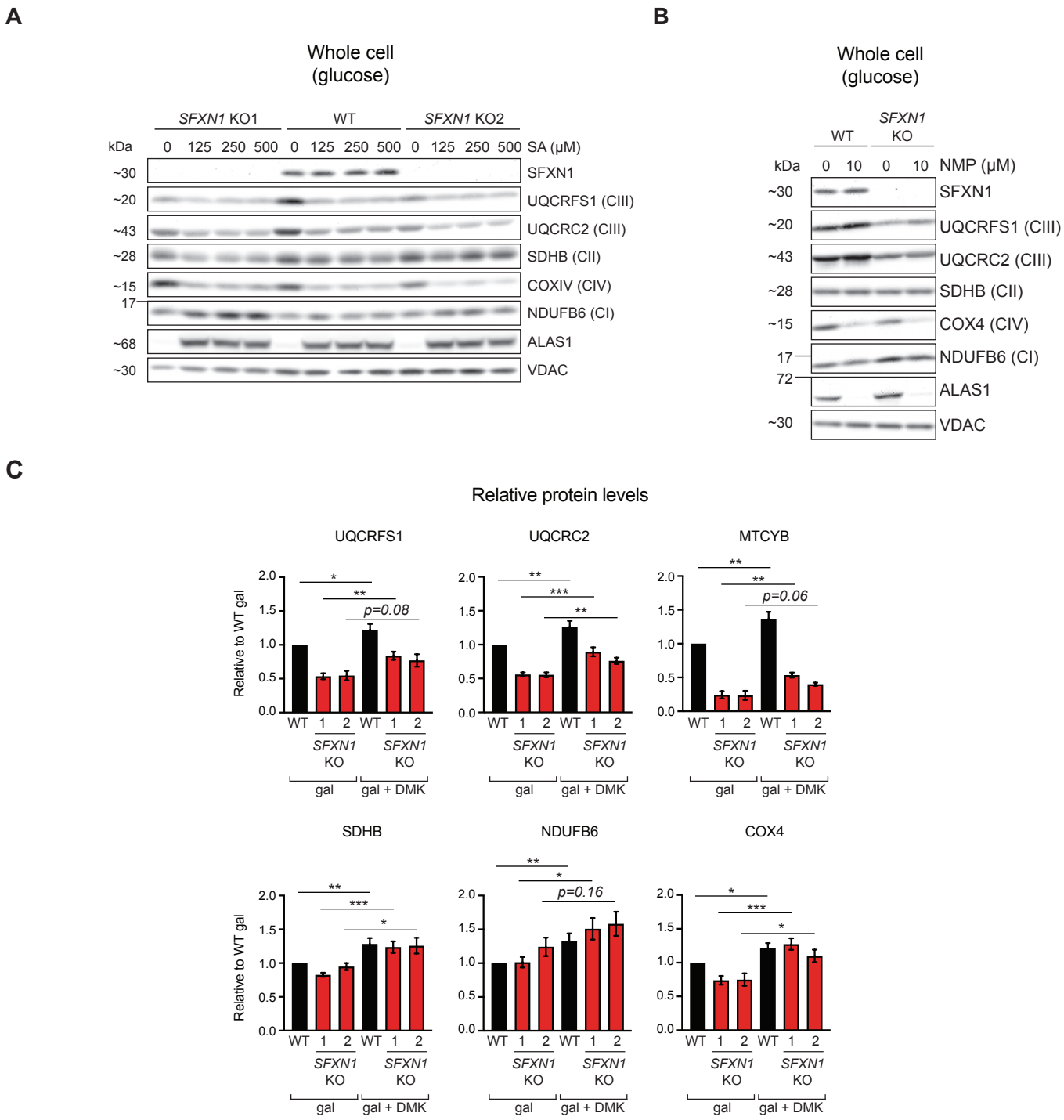


Figure S7, related to Figure 7. Effect of heme biosynthetic inhibitors or DMK supplementation on steady-state abundance of respiratory complex subunits.

(A) Immunoblotting for select respiratory complex subunits using cell lysates. Cells were grown in glucose-containing media with the indicated SA concentration for 2 days. ALAS1 protein is elevated in response to SA-mediated inhibition of heme biosynthesis.

(B) Immunoblotting for select respiratory complex subunits using cell lysates. Cells were grown in glucose-containing media with 10 mM NMP for 2 days. ALAS1 protein is decreased in response to NMP-mediated inhibition of heme biosynthesis.

(C) Densitometric analysis of bands for select proteins in (H). Protein levels in WT were set to 1.0 (mean±SEM, n≥5).

**p<0.05, **p<0.01, ***p<0.001, ****p<0.0001,* unpaired Student's t-test