Supplemental Materials

Molecular Biology of the Cell

Jackson *et al.*

Supplementary Figure Legends

Supplementary Figure 1. Changes to Complex I subunits does not rely on AGK kinase activity.

(A) Mitochondrial lysates from control HEK293 cells, AGK^{KO} HEK293 cells, and AGK^{KO} HEK293 cells re-expressing WT or kinase-dead (G126E mutant) AGK were analysed by SDS-PAGE and western blotting. **(B)** Relative protein levels were quantified and are represented as mean ± SD (n=3). One sample t-test: *, $p<0.05$, **, $p<0.01$. **(C)** Mitochondria isolated from control HEK293 cells, AGK^{KO} HEK293 cells, and AGK^{KO} HEK293 cells re-expressing WT or kinase-dead (G126E mutant) AGK were solubilised in 1% digitonin containing buffer and analysed by BN-PAGE and immunoblotting with the indicated antibodies. **(D)** Mitochondrial oxygen consumption rates of control HEK293, AGK^{KO}, $AGK^{KO\ +\ WT}$, and $AGK^{KO\ +\ G126E}$ cells were measured using a Seahorse analyser. Oligomycin, FCCP, antimycin A and rotenone were added to allow for calculation of basal, maximal and non-mitochondrial respiration rates. Basal and maximal mitochondrial oxygen consumption rates were calculated for each cell line and depicted as mean ± SD (n=4). One-sample t-test: *, $p<0.05$. **(E)** ATP synthesis rates measured in digitonin permeabilized control HEK293 and AGK^{KO} cells with CI-linked (glutamate + malate or pyruvate + malate) or CII-linked (succinate) substrates in the absence of presence of inhibitors rotenone (CI) or malonate (CII). Data are depicted as mean ± SD (n=4).

Supplementary Figure 2. Analysis of Sengers syndrome patient fibroblasts.

(A) Gene Ontology (GO) enrichment analysis was performed for both patient fibroblast lines using all proteins upregulated or downregulated >1.25 fold with $p<0.05$. GO terms were ranked according to p-value. Red terms are associated with upregulated genes, blue terms are associated with downregulated genes, and grey terms are associated with up and down-regulated genes. **(B)** Cell lysates from both patient fibroblast cell

lines and three control fibroblast cell lines were analysed by SDS-PAGE and western blotting. **(C)** The fold change in mRNA expression of *PGC1α* in the patient fibroblasts compared to three control fibroblast lines was determined by RT-qPCR and is expressed as mean ± SD (n=2 for each control, n=3 for each patient). Statistical significance was determined using two-tailed unpaired t-tests comparing the combined control data (n=6) to each patient (n=3 for each patient). **(D)** Relative abundance of respiratory chain complexes (complexes I-V) in Sengers patient fibroblasts as compared to control. Mean ± 95% CI is depicted. Ratio paired t-test: *, $p<0.05$, **, $p<0.05$. **(E)** Mitochondrial oxygen consumption rates of both patient fibroblast cell lines and 3 control fibroblast cell lines were measured using a Seahorse analyser. Oligomycin, FCCP, antimycin A and rotenone were added to allow for calculation of basal, maximal and non-mitochondrial respiration rates. All data was normalised to the average control maximal respiration value. Basal and maximal mitochondrial oxygen consumption rates were calculated for each cell line (normalised to the average of the control fibroblasts) and depicted as mean ± SD (n=2).

Supplementary Figure 3. Import of MTHFD2 and SHMT2 does not rely on the TIM22 complex.

(A) [^{35}S]-MTHFD2, [^{35}S]-SHMT2, and [^{35}S]-GC1 were incubated with mitochondria isolated from control and AGK^{KO} HEK293 cells for the indicated times and in the presence or absence of membrane potential ($\Delta\psi$). Following incubation, samples were treated with proteinase K (PK) prior to analysis by SDS-PAGE and autoradiography. **(B)** The fold change in mRNA expression of *SHMT2*, *MTHFD2*, and *SFXN1* in the patient fibroblasts compared to three control fibroblast lines was determined by RT-qPCR and is expressed as mean ± SD (n=2 for each control, n=3 for each patient). Fold-changes were normalised to the average of the three controls. Statistical significance was determined using two-tailed unpaired t-tests comparing the combined

control data (n=6) to each patient (n=3 for each patient). * p<0.05, ** p<0.01, *** p<0.001.

Supplementary Figure 4. Creation of AGK^{KO} in MCF7 cells.

(A) Schematic representation of the CRISPR/Cas9 editing system used to generate the AGK^{KO} MCF7 cell line used in the study. The resulting indel is indicated.

Supplementary Figure 5. Identification of TIM22 substrates.

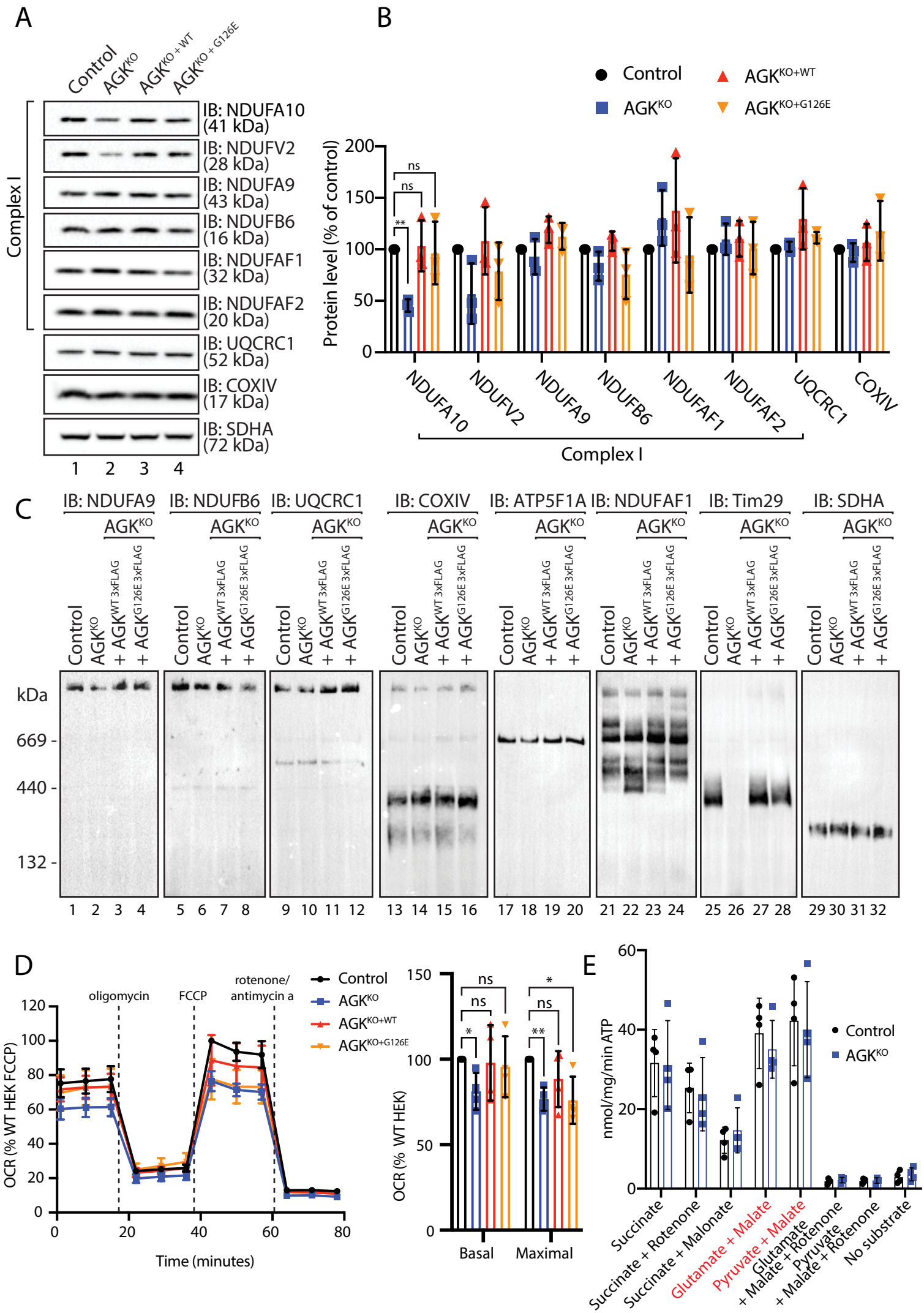
Candidate novel TIM22 complex substrates predicted from proteomics data. Downregulated proteins (log_2 fold-change >0.25, p-value <0.05) from both Sengers patient fibroblasts, AGK^{KO} and $Tim9^{MUT}$ HEK293 cells, and AGK^{KO} MCF7 cells were analysed for the presence of predicted transmembrane domains and the presence or absence of a mitochondrial targeting signal. Proteins were considered candidate TIM22 substrates if they contained >2 predicted transmembrane domains and lacked a predicted mitochondrial targeting sequence (MTS). Known TIM22 substrates are bolded, SFXNs are highlighted in yellow, and potential novel substrates are highlighted in green.

Supplementary Figure 6. The Sideroflexins.

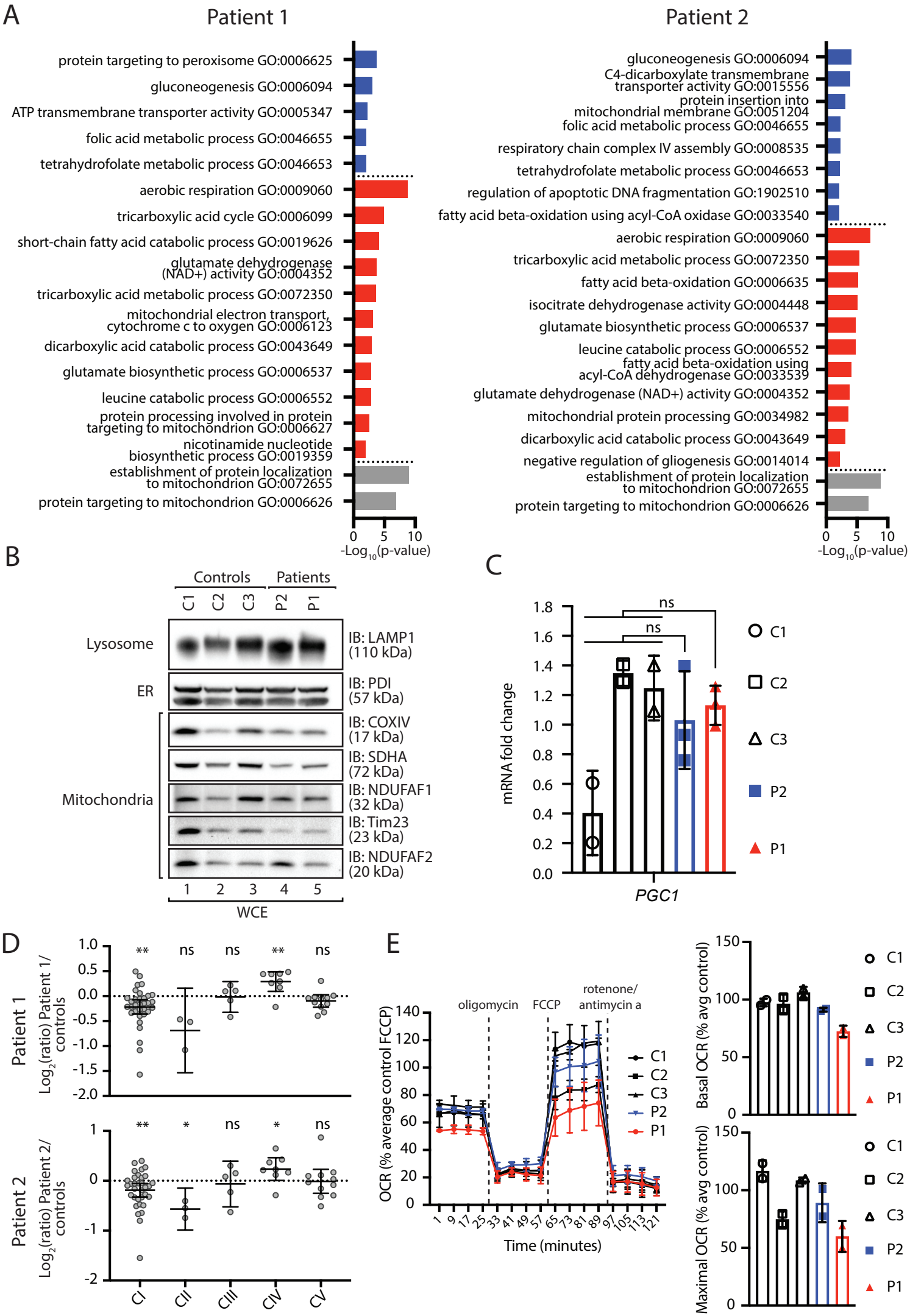
(A) Schematic representation of the CRISPR/Cas9 editing system used to generate the $SFXN1^{KO}$ HEK293 cell line used in the study. **(B)** Log_2 transformed LFQ values were depicted for the indicated proteins from control, AGK^{KO}, AGK^{KO+WT}, and $AGK^{KO+G126E}$ HEK cells. Mean ± SD is depicted (n=3). Statistical significance was determined using a one-way ANOVA and Dunnett's multiple comparisons test: *, p<0.05, **, p<0.01, ***, p<0.001.

Supplementary Figure 7. Cellular consequences of AGK dysfunction.

(A) Cell viability measured following 72 h exposure with actinonin at indicated doses in WT and AGK^{KO} cells. Data are mean ± SD (n=3). **(B)** Relative fold changes to mRNA expression of *SHMT2*, *MTHFD2*, *SLC7A11*, and *DDIT3* were determined by RT-qPCR in control and AGK^{KO} cells treated with DMSO or 50 μM actinonin. Fold-change values are normalised to DMSO treated control HEK cells. Data are mean ± SD (n=3). One sample t-test: *, $p<0.05$, **, $p<0.01$. **(C)** Cell lysates from control and AGK^{KO} HEK cells treated with DMSO or 50 μM actinonin for 24 hours were analysed by SDS-PAGE and immunoblotting. Relative protein levels were quantified and are represented as mean ± SD (n=3). One sample t-test: *, $p<0.05$, **, $p<0.01$ **(D)** Pulse SILAC analysis of newly translated mitochondria DNA encoded OXPHOS subunits. SILAC media was added following 24h treatment with chloramphenicol and analysis was performed at 1, 3 and 4-hours post SILAC media incubation. Log2 transformed heavy-peptide derived intensities were plotted relative to control. Statistical analysis was performed on each time point (n=3) compared to control WT using a t-test and FDR-1% with no significance recorded. Data are depicted as mean ± SD.

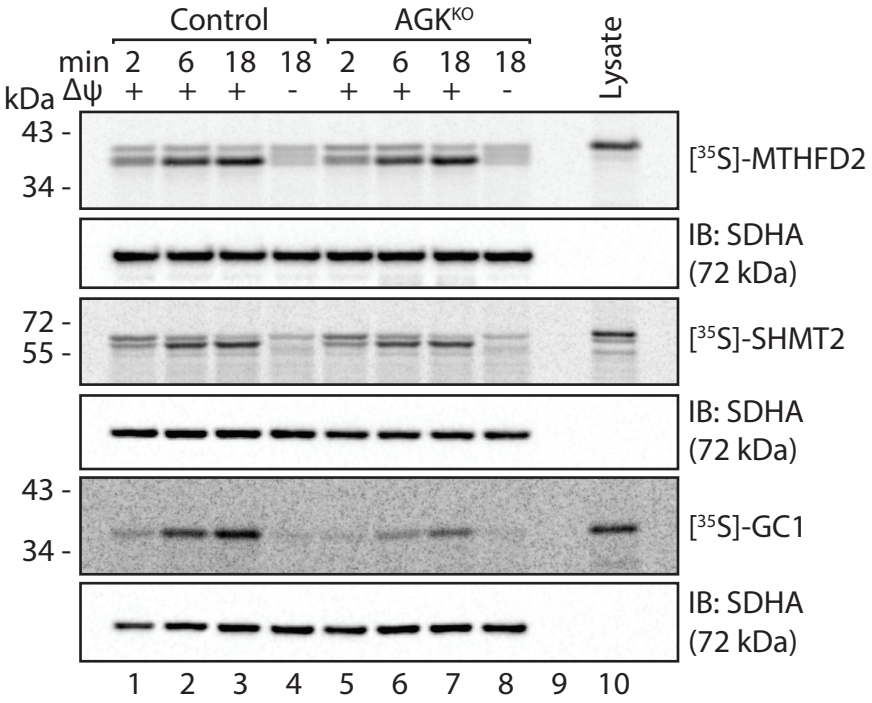


Jackson et al., Supplementary Figure 1 - Revised Version 2



Jackson et al., Supplementary Figure 2 - Revised Version 2

A


Control
AGK^KO
min 2 6 18 18 2 6 18 18
kDa Δψ + + + - + + + -
Lysate
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34 -
[35S]-MTHFD2
IB: SDHA (72 kDa)
72 -
55 -
[35S]-SHMT2
IB: SDHA (72 kDa)
43 -
34 -
[35S]-GC1
IB: SDHA (72 kDa)
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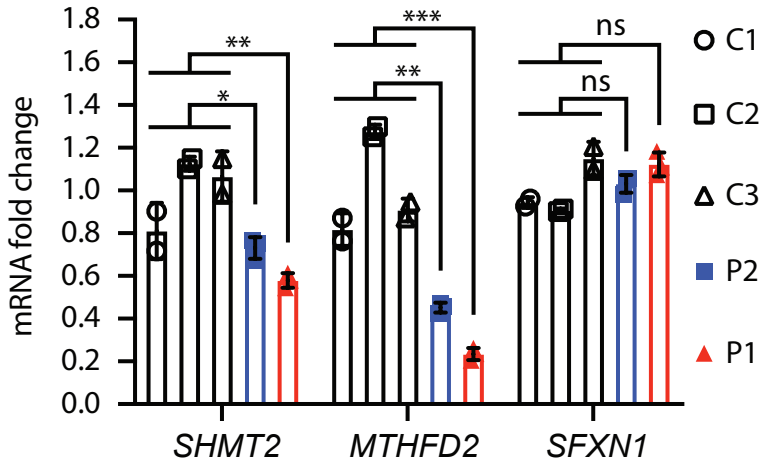
B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1.0
0.8
0.6
0.4
0.2
0.0
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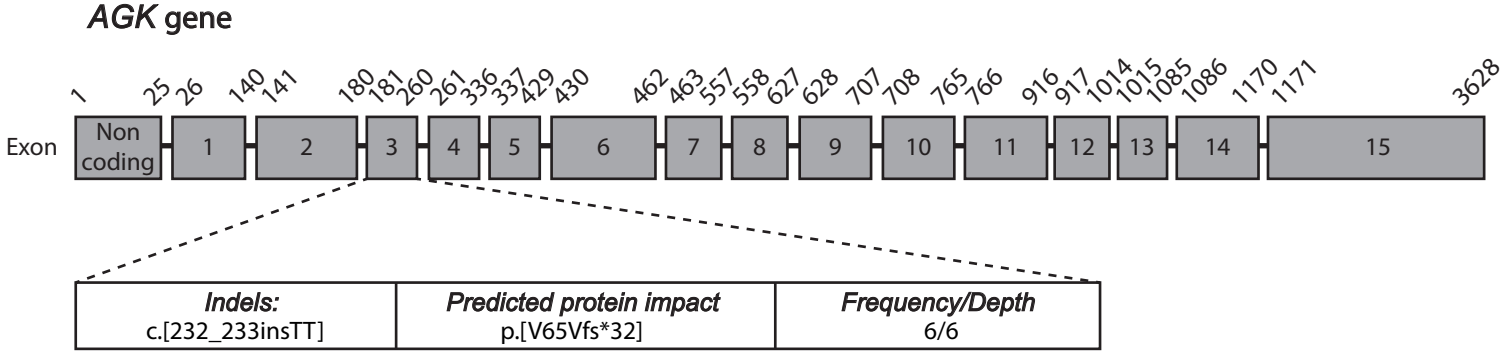
*
***
**
ns
ns
C1
C2
C3
P2
P1
SHMT2
MTHFD2
SFXN1


AGK gene
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708
765
766
916
917
1014
1015
1085
1086
1170
1171
3628
Exon
Non coding
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2
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14
15
Indels:
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Predicted protein impact
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Frequency/Depth
6/6

AGK^{KO} HEK293

GENE NAME	FOLD CHANGE	TM DOMAINS	MTS
SLC25A21	-2.37385	4	No
SLC25A4	-2.03936	6	No
SFXN2	-1.73885	4	No
SLC25A15	-1.47181	4	No
SLC25A1	-1.46192	5	Yes
SLC25A25	-1.2543	4	No
SLC25A5	-1.22732	4	No
SLC25A24	-1.1808	4	No
SLC25A19	-0.9928	6	No
TMEM126B	-0.988847	4	No
SLC25A6	-0.968911	6	No
SLC25A22	-0.93888	6	No
SLC25A10	-0.803633	3	No
NDUFA11	-0.775869	3	No
SLC25A20	-0.587364	6	No
SLC25A11	-0.573032	6	No
SFXN3	-0.516064	4	No
SFXN4	-0.508841	5	No
SLC25A12	-0.43222	3	No
OSGEPL1	-0.366842	2	Yes
SFXN1	-0.340591	3	No
TYSND1	-0.32607	2	No
PTCD1	-0.278274	2	No
TIMM17B	-0.271266	3	No
GHITM	-0.258382	8	Yes

TIM9^{MUT} HEK293

GENE NAME	FOLD CHANGE	TM DOMAINS	MTS
SLC25A4	-2.23093	6	No
SLC25A22	-2.22961	6	No
SLC25A5	-1.81683	4	No
SLC25A32	-1.6221	6	No
SLC25A21	-1.59656	4	No
SLC25A6	-1.52261	6	No
SFXN2	-1.38191	4	No
NDUFA11	-0.95851	3	No
MT-ND4	-0.943498	13	No
SLC25A19	-0.933768	6	No
SLC25A11	-0.933644	6	No
MT-ND5	-0.914134	18	No
SLC25A3	-0.728132	6	Yes
SLC25A10	-0.631883	3	No
SLC25A15	-0.584677	4	No
NDUFC2	-0.580933	2	No
SLC25A20	-0.470694	6	No
MT-ND1	-0.458689	8	No
SFXN3	-0.445435	4	No
MT-CO1	-0.444707	12	No
GPAM	-0.42632	2	No
SFXN1	-0.403746	3	No
NDUFA9	-0.393661	2	Yes
MT-CO2	-0.379013	2	No
DHCR24	-0.342808	3	No
TIMM17A	-0.321624	3	No
SLC25A1	-0.317672	5	Yes
STARD7	-0.285199	2	Yes
TIMM23	-0.258502	4	No

AGK^{KO} MCF7

GENE NAME	FOLD CHANGE	TM DOMAINS	MTS
SLC25A29	-2.75039	2	No
SLC25A12	-2.31273	3	No
SLC25A25	-1.73049	4	No
PLGRKT	-1.51383	2	No
SLC25A40	-1.48649	6	No
SLC25A15	-1.43462	4	No
SFXN2	-1.38658	4	No
MTFP1	-1.3043	3	No
FUNDC2	-1.00328	3	No
SLC25A4	-0.936395	6	No
SLC25A19	-0.798908	6	No
ABCB8	-0.797518	4	Yes
TMEM223	-0.756421	2	Yes
TMEM126B	-0.701326	4	No
SLC25A20	-0.681419	6	No
OSGEPL1	-0.656896	2	Yes
SLC25A5	-0.566826	4	No
SLC25A22	-0.538475	6	No
NNT	-0.535522	16	Yes
SLC25A1	-0.485948	5	Yes
NDUFA9	-0.459034	2	Yes
TIMM17B	-0.455844	3	No
NDUFA11	-0.405849	3	No
GPD2	-0.399118	2	Yes
TIMM23	-0.371304	4	No
TOMM22	-0.364568	2	No
MT-CO1	-0.347233	12	No
SLC25A36	-0.332314	2	No
MT-CO2	-0.32247	2	No
SFXN4	-0.268023	5	No

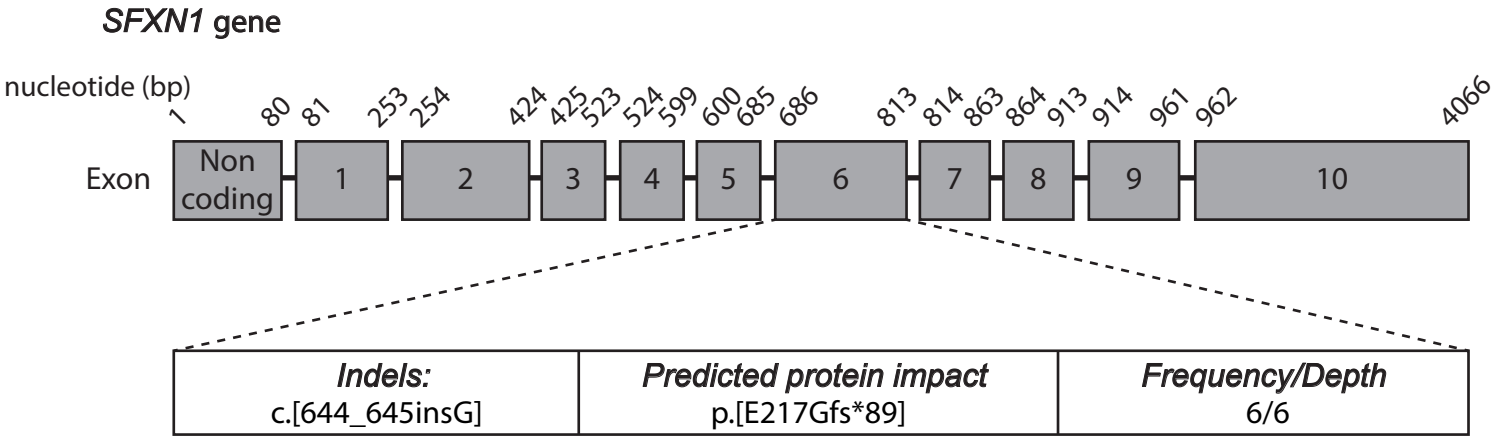
Sengers patient 1

GENE NAME	FOLD CHANGE	TM DOMAINS	MTS
SLC25A1	-1.29154	5	Yes
SLC25A24	-1.12644	4	No
TMEM126A	-0.858381	4	No
TIMMDC1	-0.81462	4	No
TCIRG1	-0.748076	9	No
SLC25A11	-0.720854	6	No
SLC25A3	-0.570413	6	Yes
SLC25A6	-0.543805	6	No
PHB2	-0.543002	2	Yes
TMEM205	-0.528653	4	No
RDH11	-0.453039	2	No
SFXN1	-0.396356	3	No

Sengers patient 2

GENE NAME	FOLD CHANGE	TM DOMAINS	MTS
SLC25A24	-1.30442	4	No
SLC25A1	-1.25611	5	Yes
TMEM126A	-1.05873	4	No
SLC25A22	-0.90056	6	No
TMEM205	-0.875169	4	No
SLC25A11	-0.704802	6	No
SFXN1	-0.655487	3	No
SLC25A12	-0.647718	3	No
ABCB8	-0.439109	4	Yes
RDH14	-0.430247	3	Yes
BAX	-0.340385	2	No
SLC25A6	-0.284163	6	No

A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913
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962
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Exon
Non coding
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Predicted protein impact
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Frequency/Depth
6/6

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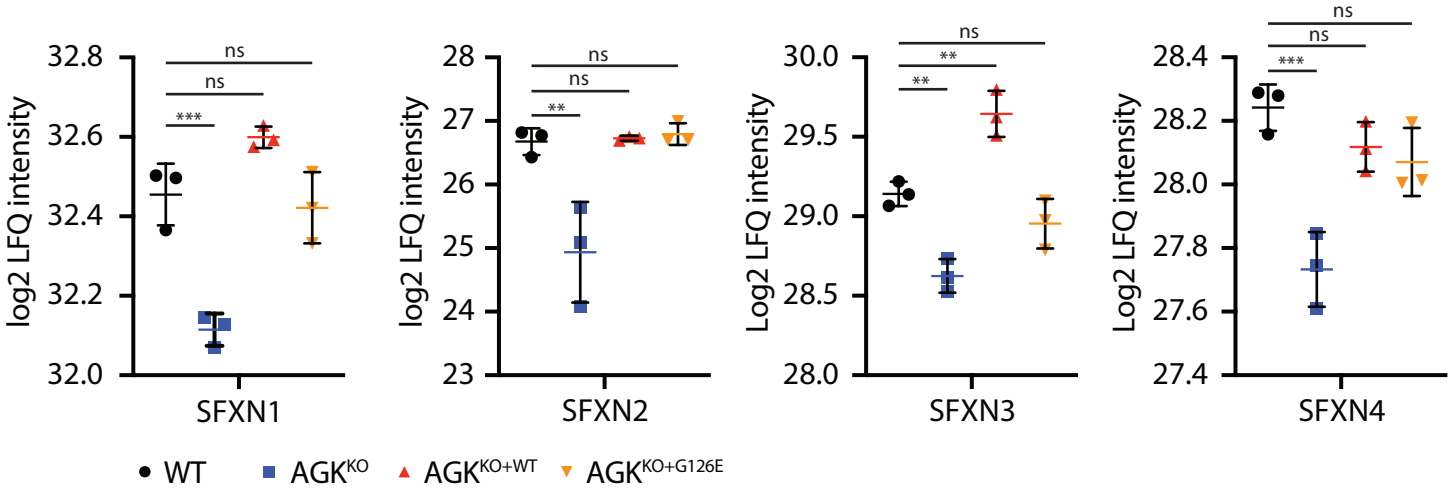
log2 LFQ intensity
Log2 LFQ intensity
SFXN1
SFXN2
SFXN3
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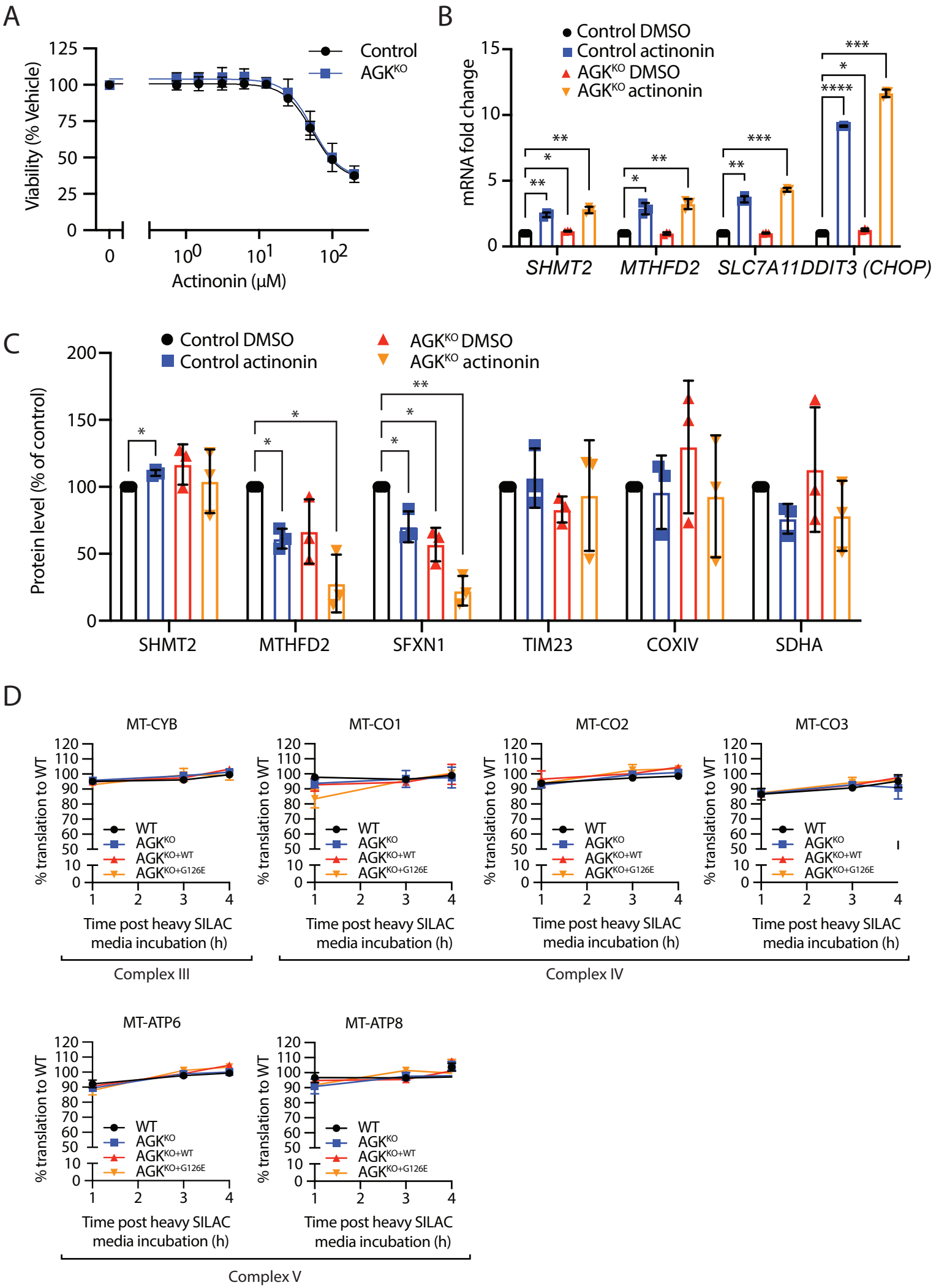
ns
***
**
WT
AGK^KO
AGK^KO+WT
AGK^KO+G126E



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