Cell Stem Cell, Volume *28*

Supplemental Information

CRISPR-Mediated Induction of Neuron-Enriched Mitochondrial Proteins Boosts Direct Glia-to-Neuron Conversion

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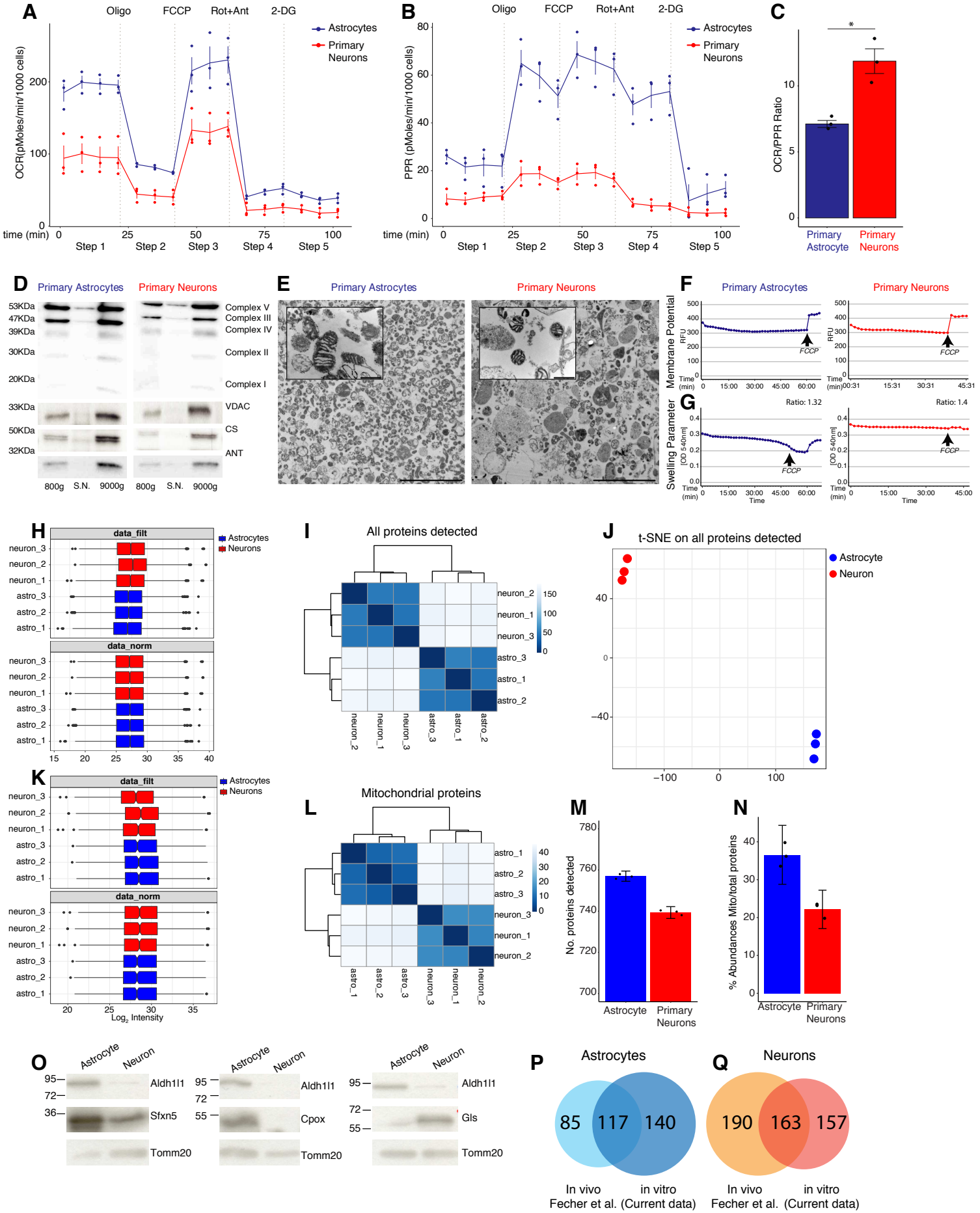
Figure S1


A
Oligo
FCCP
Rot+Ant
2-DG
Astrocytes
Primary Neurons
OCR(pMoles/min/1000 cells)
time (min)
Step 1
Step 2
Step 3
Step 4
Step 5
B
PPR (pMoles/min/1000 cells)
C
OCR/PPR Ratio
Primary Astrocyte
Primary Neurons
D
Primary Astrocytes
Primary Neurons
53KDa
47KDa
39KDa
30KDa
20KDa
33KDa
50KDa
32KDa
Complex V
Complex III
Complex IV
Complex II
Complex I
VDAC
CS
ANT
800g
S.N.
9000g
E
F
Membrane Potential
RFU
FCCP
G
Swelling Parameter
Ratio: 1.32
Ratio: 1.4
H
data_filt
data_norm
Astrocytes
Neurons
I
All proteins detected
J
t-SNE on all proteins detected
Astrocyte
Neuron
K
Log2 Intensity
L
Mitochondrial proteins
M
No. proteins detected
N
% Abundances Mito/total proteins
O
Aldh1l1
Sfxn5
Cpox
Gls
Tomm20
P
Astrocytes
85
117
140
In vivo Fecher et al.
in vitro (Current data)
Q
Neurons
190
163
157

SUPPLEMENTAL MATERIAL

Figure S1, related to Figure 1. Astrocytes and neurons from cerebral cortex differ in mitochondrial function

(**A-B**) Longitudinal traces of extracellular flux analysis measured by Seahorse XF analyzer, comparing the Oxygen Consumption Rate (**A**) and the Proton Production Rate (**B**) of astrocytes (blue) versus neurons (red) over time, after challenging the cells with different ETC inhibitors. Values are normalized per 1000 cells. Each time point is shown as mean ± SD. n=3 experimental batches for each group.

(C) Barplot showing OCR/PPR ratio in cultures of primary astrocytes versus neurons, as measured by Seahorse XF analyzer. * $p \leq 0.05$. n=3 for each group.

(D) Immunoblot detection of mitochondrial proteins in different fractions (800g, nuclear; S.N., cytosolic; 9000g, mitochondria and other organelles) isolated from astrocytes or neurons.

(E) Electron Microscopy images of mitochondria isolated from astrocyte and neuron cultures. Scale bar: 5µm. Magnifications scale bar: 500nm.

(**F**) Graphs showing the membrane potential of mitochondria isolated from astrocytes (*upper panel*) and neurons (*lower panel*) measured by Rhodamine 123 assay, indicating their healthy functional state.

(**G**) Graphs showing the swelling parameter of mitochondria isolated from astrocytes (*upper panel*) and neurons (*lower panel*) by absorbance at 540nm indicating their healthy functional state.

(H) Boxplot depicting abundances of all proteins before (*upper panel*) and after (*lower panel*) normalization.

(I) Unsupervised cluster analysis of the samples considering all quantified proteins.

(J) *t*-SNE of the samples, based on all proteins after normalization.

(K) Boxplot depicting abundances of mitochondrial proteins selected according to mitoCarta before (*upper panel*) and after (*lower panel*) normalization.

(L) Unsupervised cluster analysis of the samples considering only mitochondrial proteins.

(M) Barplot showing the number of mitochondrial proteins identified by mass spec in astrocytes (blue) and neurons (red). Each dot represents a biological replicate.

(N) Barplot depicting the percentage of mitochondrial protein abundance over total protein abundance in astrocytes (blue) and neurons (red). Each dot represents a biological replicate.

(**O**) Western blots of total lysates from cultured astrocytes or neurons confirming the selective cell enrichment by high amounts of Adlh1l1 in astrocyte lysates, equal mitochondrial protein loading by Tomm20 and the higher amount of Sfxn5 and Cpox in astrocytes and Gls in neurons.

(**P,Q**) Venn Diagrams of all astrocyte-enriched (**I**) and neuron-enriched (**J**) mitochondrial proteins detected by Fecher et al. and our analysis showing a high degree of overlap given that mitochondrial proteins were isolated at different stages (adult versus postnatal), from different regions (cerebellum versus cortex) and in different conditions *(in vivo* versus *in vitro*).

Figure S2

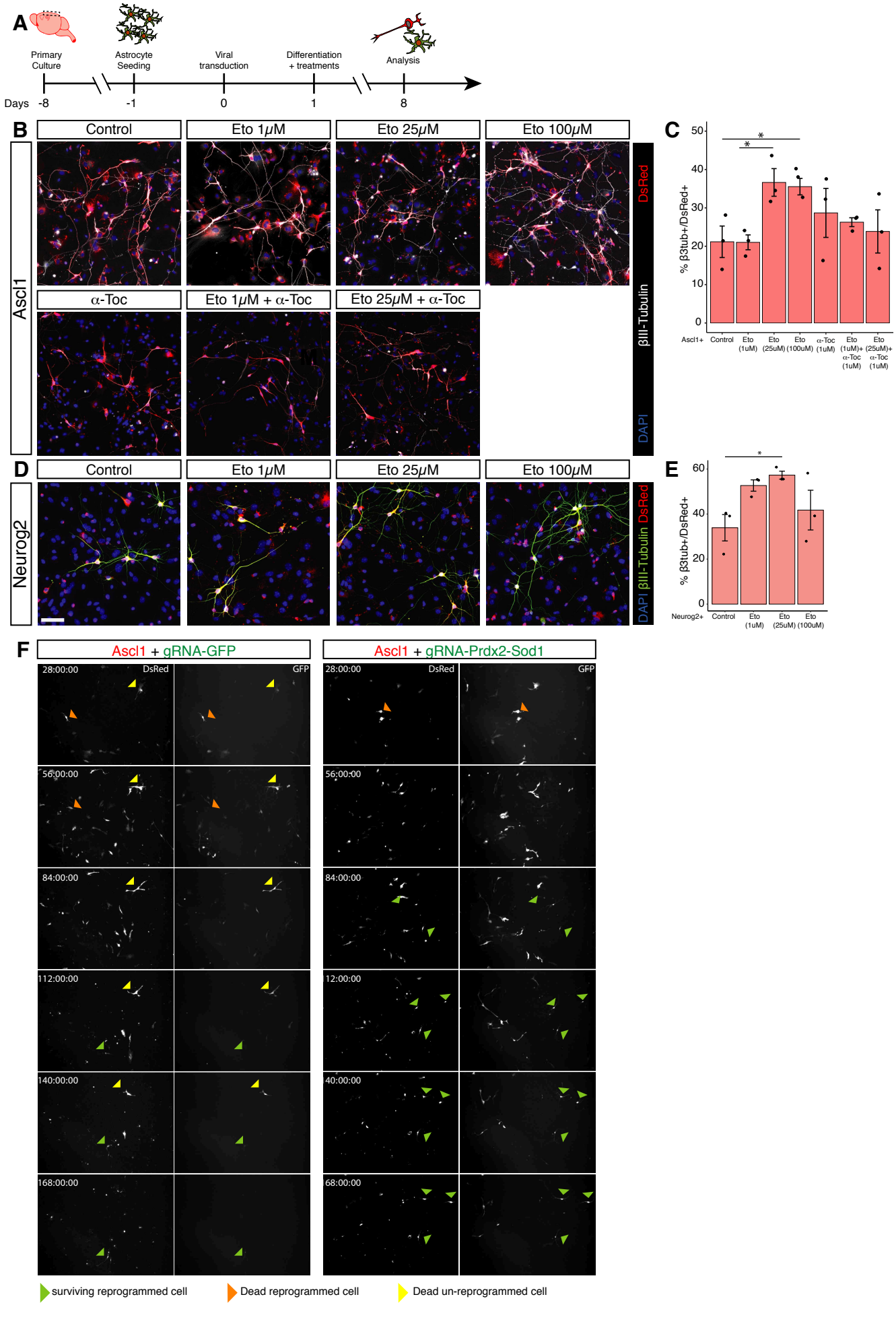

A
Primary Culture
Astrocyte Seeding
Viral transduction
Differentiation + treatments
Analysis
Days -8 -1 0 1 8
B
Control
Eto 1μM
Eto 25μM
Eto 100μM
α-Toc
Eto 1μM + α-Toc
Eto 25μM + α-Toc
Ascl1
DsRed
βIII-Tubulin
DAPI
C
% β3tub+/DsRed+
Ascl1+ Control Eto (1uM) Eto (25uM) Eto (100uM) α-Toc (1uM) Eto (1uM)+ α-Toc (1uM) Eto (25uM)+ α-Toc (1uM)
D
Neurog2
E
Neurog2+ Control Eto (1uM) Eto (25uM) Eto (100uM)
F
Ascl1 + gRNA-GFP
Ascl1 + gRNA-Prdx2-Sod1
28:00:00
56:00:00
84:00:00
112:00:00
140:00:00
168:00:00
surviving reprogrammed cell
Dead reprogrammed cell
Dead un-reprogrammed cell

Figure S2, related to Figure 1 and 4. Etomoxir treatment improves direct neuronal reprogramming.

(**A**) Schematic drawing of the experimental setup.

(B) Micrographs showing the Ascl1-transduced cells (DsRed+) that are βIII-tubulin+ neurons (in white), nuclei labeled in DAPI (Blue), in the treatment conditions indicated. Scale bar: 50µm.

(C) Histogram depicting the percent of βIII-tubulin+ cells amongst Ascl1-transduced $DsRed^+$ cells at 8 DPI. Data are shown as mean±SEM. Each dot represents a biological replicate (n=3 for experimental condition). *$p \leq 0.05$

(**D**) Micrographs showing the efficiency of neuronal conversion in upon Neurog2 expression together with treatment of different concentration of Etomoxir. Reprogrammed neurons (DsRed+) are βIII-tubulin+ (in white). Nuclei labeled in DAPI (Blue). Scale bar: 50µm.

(**E**) Histogram depicting the percentage of βIII-tubulin+ cells amongst Neurog2-transduced $DsRed^+$ cells at 8 DPI. Data are shown mean±SEM. Each dot represents a biological replicate (n=3 for experimental condition). *$p \leq 0.05$

(F) Example of fluorescent pictures acquired during the imaging and used for analysis (related to **Figure 4**).

Figure S3

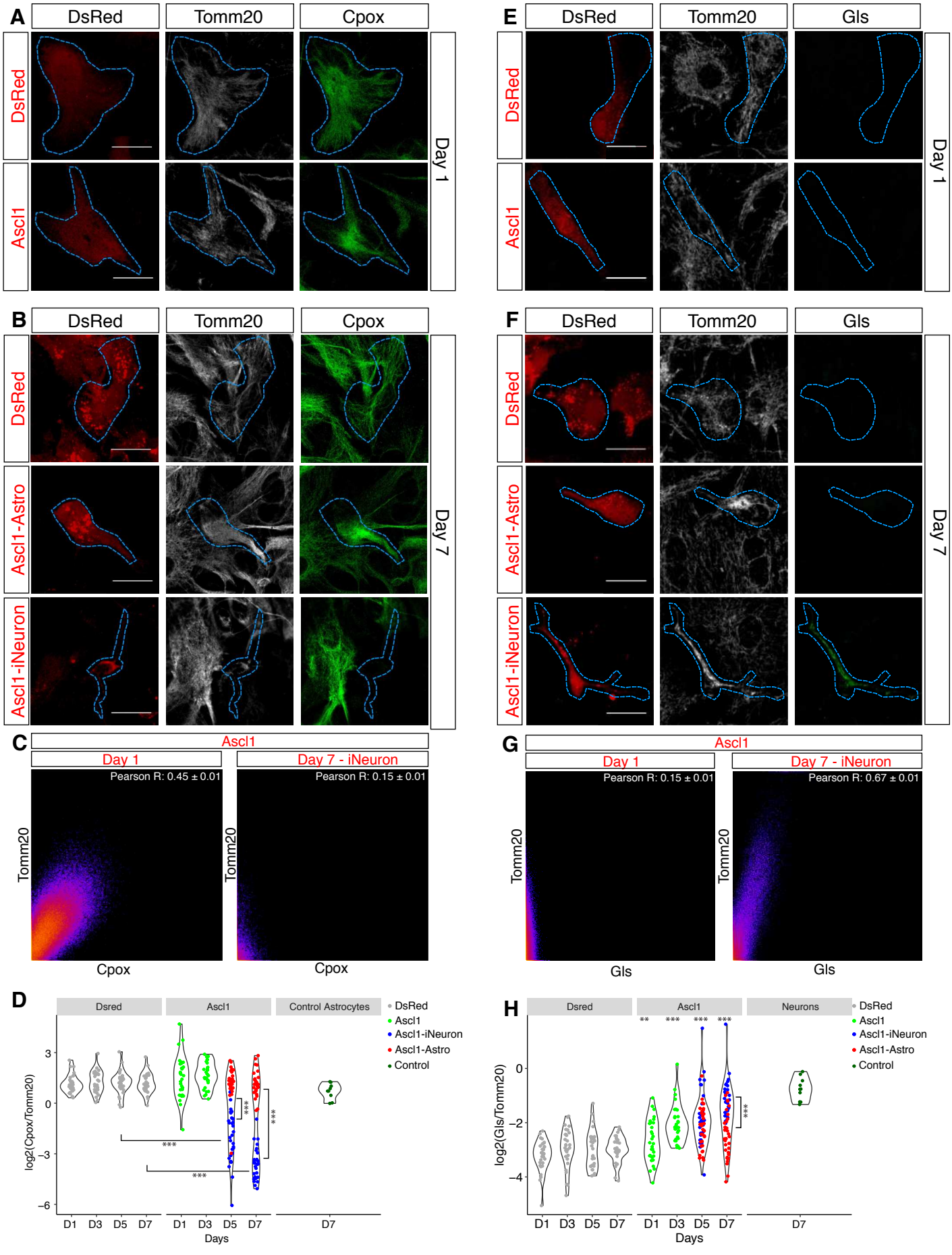

A
DsRed
Tomm20
Cpox
DsRed
Ascl1
Day 1
E
DsRed
Tomm20
Gls
DsRed
Ascl1
Day 1
B
DsRed
Tomm20
Cpox
DsRed
Ascl1-Astro
Ascl1-iNeuron
Day 7
F
DsRed
Tomm20
Gls
DsRed
Ascl1-Astro
Ascl1-iNeuron
Day 7
C
Ascl1
Day 1
Pearson R: 0.45 ± 0.01
Day 7 - iNeuron
Pearson R: 0.15 ± 0.01
Tomm20
Cpox
G
Ascl1
Day 1
Pearson R: 0.15 ± 0.01
Day 7 - iNeuron
Pearson R: 0.67 ± 0.01
Tomm20
Gls
D
Dsred
Ascl1
Control Astrocytes
log2(Cpox/Tomm20)
Days
DsRed
Ascl1
Ascl1-iNeuron
Ascl1-Astro
Control
H
Dsred
Ascl1
Neurons
log2(Gls/Tomm20)
Days
DsRed
Ascl1
Ascl1-iNeuron
Ascl1-Astro
Control

Figure S3, related to Figure 2. Mitochondrial protein changes during astrocyte-to-neuron reprogramming

(**A, B**) Micrographs showing immunostainings of the astrocyte-enriched mitochondrial protein Cpox in astrocytes transduced with DsRed (control) or Ascl1-ires-DsRed at 1 (**A**) or 7 (**B**) DPI as indicated. Mitochondria are identified by the expression of Tomm20. Scale bar: 20µm.

(**C**) Example of scatter plot of the pixel intensity correlation between Tomm20 and Cpox in Ascl1-transduced cells at 1 (*left panel*) and 7 (*right panel*) DPI. Pearson's coefficient as average of 3 cells/biological replicate; n=3 biological replicates.

(**D**) Violin plot depicting the log2-ratio of the intensity of the expression of Cpox versus Tomm20 over time (D1, D3, D5, D7) and in cortical astrocyte cultures at day 7. Each dot represents 1 analyzed cell. 10 cells analyzed per biological replicate, each condition. n=3 biological replicates; ***$p \leq 0.001$.

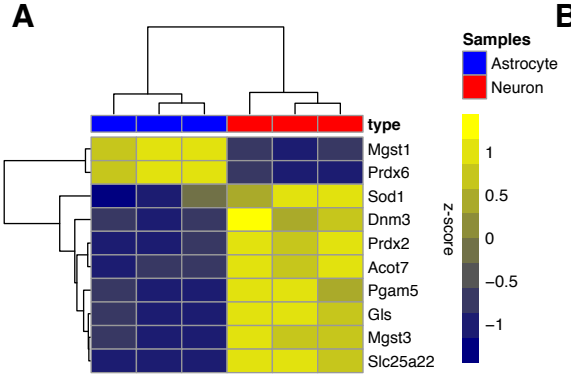
(**E, F**) Micrographs showing the expression of the neuron-specific mitochondrial protein Prdx2 in astrocytes transduced with DsRed (control) or Ascl1-ires-DsRed at 1 (**E**) and 7 (**F**) DPI. Mitochondria are identified by the expression of Tomm20. Scale bar: 20µm.

(**G**) Example of scatter plot of the pixel intensity correlation between Tomm20 and Gls in Ascl1-transduced cells at 1 (left panel) and 7 (right panel) DPI. Pearson's coefficient as average of 3 cells/biological replicate; n=3 biological replicates.

(**H**) Violin plot depicting the log2-ratio of the intensity of the expression of Gls versus Tomm20 over time (D1, D3, D5, D7) and in E14 cortex-derived cultures at 7 days in vitro. Each dot represents 1 analyzed cell. 10 cells analyzed per biological replicate, each condition. n=3 biological replicates; ***$p \leq 0.001$.

Figure S4

A

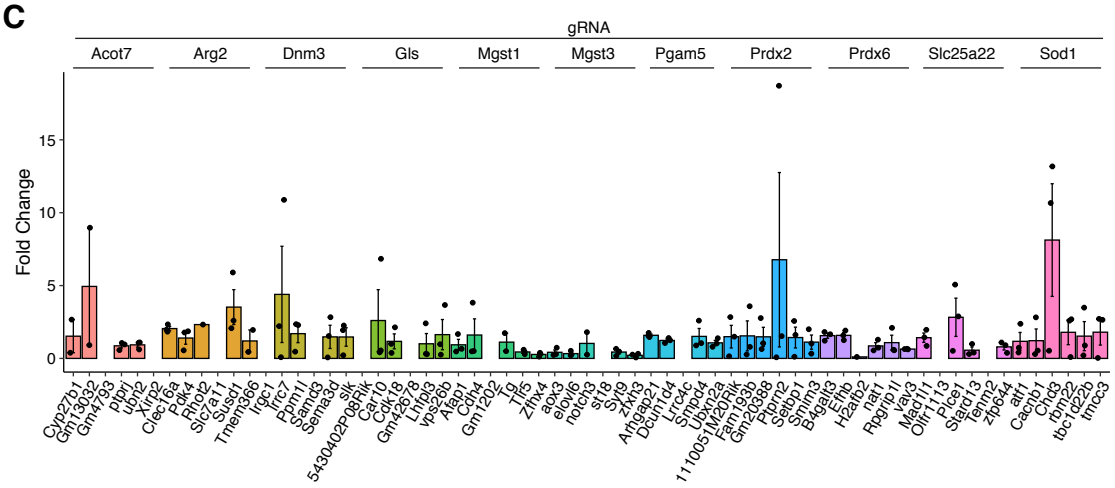


B

Genes	gRNA-ctl	gRNA-Prdx2-Sod1	gRNA-Arg2-Gls	gRNA-AD
GAPDH	27	25,88333333	25,29666667	27,04666667
Prdx2	30,06666667	25,85333333		
Sod1	26,91333333	24,23		
Arg2	40,54333333		28,22333333	29,36666667
Gls	34,59666667		31,34333333	
Dnm3	37,68			33,71666667

Genes	gRNA-ctl	gRNA-Acot7	gRNA-Slc25a22	gRNA-Pgam5	gRNA-Prdx6	gRNA-Mgst1	gRNA-Mgst3
gapdh	27,46	26,9225	30,845	29,4725	27,8425	28,215	29,575
Acot7	33,745	30,38666667					
Mgst1	33,325					32,02	
Mgst3	35,15666667						36,8
Pgam5	33,73666667			35,69333333			
Prdx6	32,8975				32,35666667		
Pgam5	33,73666667			35,69333333			
Slc25a22	35,565		36,63				

C



D

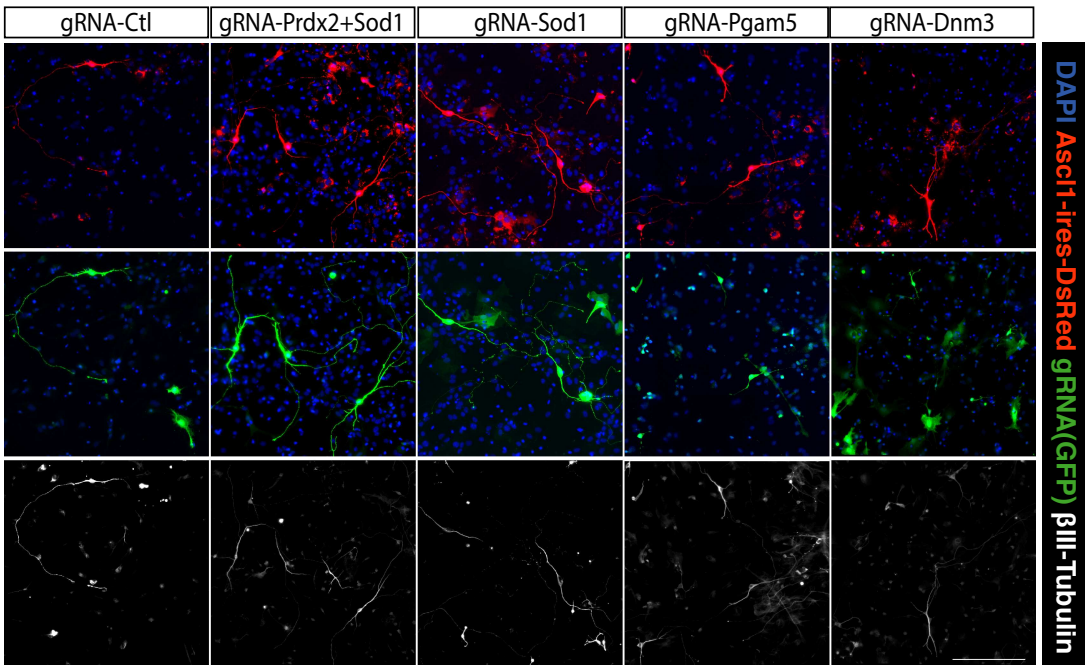


Figure S4, related to Figure 3. Characterization of selected candidates

(**A**) Unsupervised heatmap depicting the relative expression of the selected candidates.

(**B**) Graphs depicting the log2-normalized abundance of the astrocyte-enriched (blue dots) and neuron-enriched (red dots) candidates, as analyzed in Figure 2. Each dot represents a biological replicate.

(**C**) Real Time quantitative PCR (RT-qPCR) showing the fold change of putative off-targets following the transfection of gene-specific gRNA. Data are shown as fold change over the gRNA-scramble control (mean ± SEM). None of this is significant over Paired t-test used. control. n=3 biological replicates for each group.

(**D**) Single channel immunofluorescence images showing reprogrammed neurons (βIII-tubulin^{+}-DsRed^{+}-GFP^{+}) transfected by Ascl1-ires-DsRed (red) and different STAgR constructs (green). Scale bar: 100μm.