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Supplemental information

Direct neuronal reprogramming of NDUFS4 patient cells identifies the unfolded protein response as a novel general reprogramming hurdle

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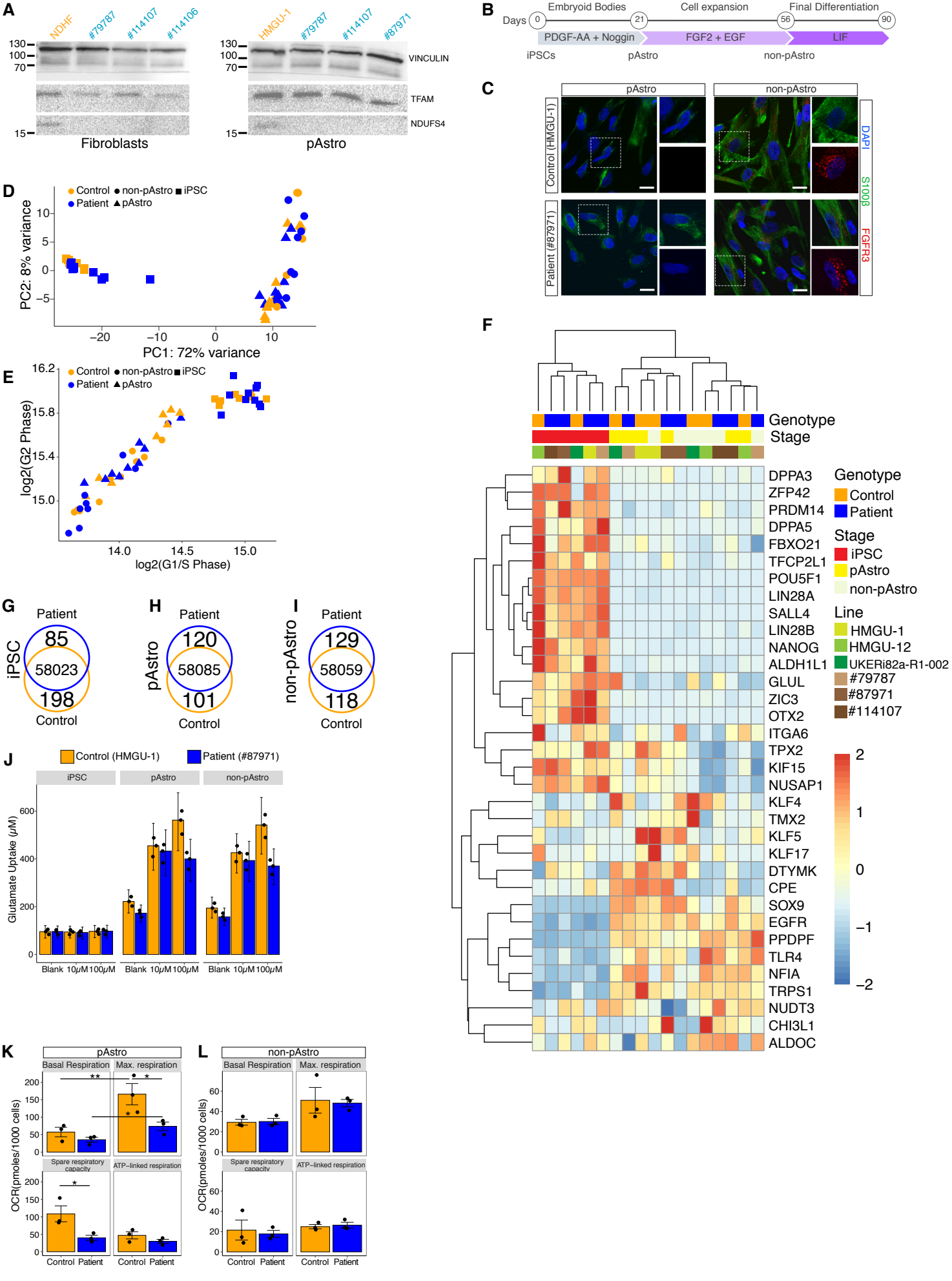
Figure S1


A
NDHF
#79787
#114107
#114106
HMGU-1
#87971
130
100
70
15
VINCULIN
TFAM
NDUFS4
Fibroblasts
pAstro
B
Embryoid Bodies
Cell expansion
Final Differentiation
Days
0
21
56
90
PDGF-AA + Noggin
FGF2 + EGF
LIF
iPSCs
pAstro
non-pAstro
C
pAstro
non-pAstro
Control (HMGU-1)
Patient (#87971)
DAPI
S100β
FGFR3
D
Control
non-pAstro
iPSC
Patient
pAstro
PC2: 8% variance
PC1: 72% variance
E
log2(G2 Phase)
log2(G1/S Phase)
F
Genotype
Stage
DPPA3
ZFP42
PRDM14
DPPA5
FBXO21
TFCP2L1
POU5F1
LIN28A
SALL4
LIN28B
NANOG
ALDH1L1
GLUL
ZIC3
OTX2
ITGA6
TPX2
KIF15
NUSAP1
KLF4
TMX2
KLF5
KLF17
DTYMK
CPE
SOX9
EGFR
PPDPF
TLR4
NFIA
TRPS1
NUDT3
CHI3L1
ALDOC
Genotype
Control
Patient
Stage
iPSC
pAstro
non-pAstro
Line
HMGU-1
HMGU-12
UKERi82a-R1-002
#79787
#87971
#114107
2
1
0
−1
−2
G
Patient
iPSC
85
58023
198
Control
H
Patient
pAstro
120
58085
101
Control
I
Patient
non-pAstro
129
58059
118
Control
J
Control (HMGU-1)
Patient (#87971)
iPSC
pAstro
non-pAstro
Glutamate Uptake (μM)
Blank
10μM
100μM
K
pAstro
Basal Respiration
Max. respiration
Spare respiratory capacity
ATP−linked respiration
OCR(pmoles/1000 cells)
Control
Patient
L
non-pAstro
Basal Respiration
Max. respiration
Spare respiratory capacity
ATP−linked respiration
OCR(pmoles/1000 cells)
Control
Patient

Supplementary Fig. S1 (related to Fig. 1): Differentiation of control and NDUFS4-patient iPSCs into astrocytes.

(**A**) Western Blots depicting the expression of NDUFS4 in control and patient fibroblasts (left) and pAstros (right). Vinculin was used as loading control; TFAM was used to detect the presence of mitochondrial proteins in the lysate.

(**B**) Scheme of the differentiation protocol used to obtain iPSC-derived astrocytes.

(**C**) Micrographs of control and patient cells at two different stages of astrocyte differentiation. Scale bars = 50 μm.

(**D**) Principal Component (PC) analysis of RNA-seq data from control and patient cells at different stages (iPSC, pAstros, non-pAstros). Control samples shown in orange; patient samples shown in blue. n = 3 independent culture batches lines analyzed per each donor. n=3 independent culture batches per stage.

(**E**) Scatter plot depicting the samples according to their cell cycle phase. Each dot represents a sample.

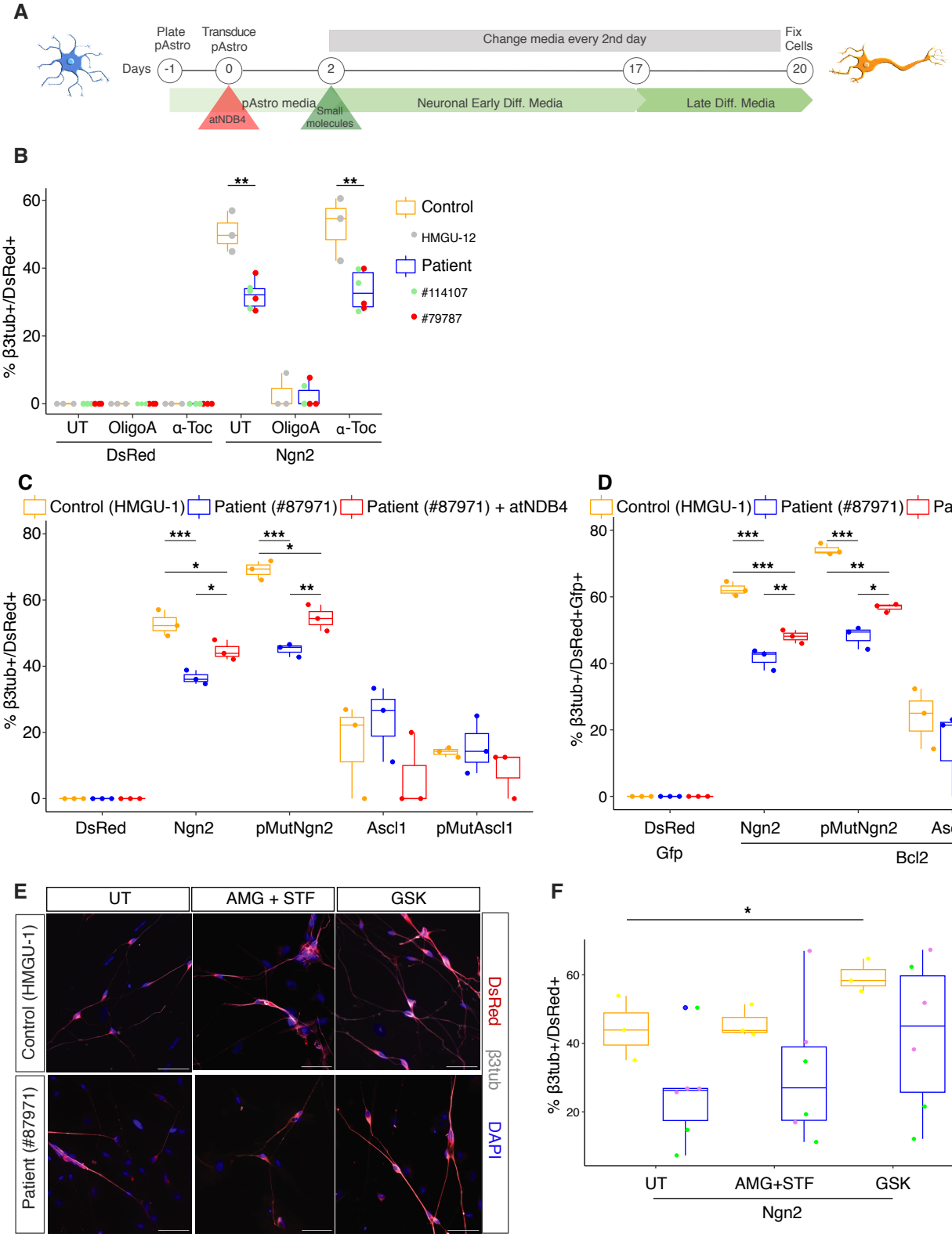
(**F**) Heatmap analysis of different pluripotent and astrocyte markers across control and patient samples at different stages of differentiation (iPSC, pAstros and non-pAstros). The color scale indicates Z-score.

(**G, H, I**) Venn diagram showing the genes differentially expressed between patient and control iPSCs (**G**), pAstros (**H**) and non-pAstros (**I**). Genes were considered upregulated in patient if log2(FC)> 1 and pvalue <0.01 or upregulated in control if log2(FC)> 1 and pvalue <0.01.

(**J**) Barplot depicting the glutamate uptake of control (orange) and patient (blue) iPSCs, pAstros and non-pAstros. Each dot represents a biological replicate. n=3 independent culture batches. Data are shown as mean ± SEM.

(**K, L**) Barplots depicting various oxygen consumption rate (OCR) in basal conditions and upon treatment with different small molecules (see material and methods) in control (orange) and patient (blue) pAstros (**K**) and non-pAstros (**L**). Each dot represents an independent culture batch (n=3). Data are shown as mean ± SEM. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$.

Figure S2


A
Plate pAstro
Transduce pAstro
Change media every 2nd day
Fix Cells
Days
-1
0
2
17
20
pAstro media
atNDB4
Small molecules
Neuronal Early Diff. Media
Late Diff. Media
B
% β3tub+/DsRed+
Control
HMGU-12
Patient
#114107
#79787
UT
OligoA
α-Toc
DsRed
Ngn2
C
Control (HMGU-1)
Patient (#87971)
Patient (#87971) + atNDB4
% β3tub+/DsRed+
DsRed
Ngn2
pMutNgn2
Ascl1
pMutAscl1
D
% β3tub+/DsRed+Gfp+
Gfp
Bcl2
E
UT
AMG + STF
GSK
Control (HMGU-1)
Patient (#87971)
DsRed
β3tub
DAPI
F
% β3tub+/DsRed+
Control
HMGU-1
Patient
#79787
#87971
AMG+STF

Supplementary Fig. S2 (related to Fig. 2): Pharmacological treatments and genetic rescues in pAstros cells

(**A**) Experimental design for either treatment with small molecules or for the genetic rescue.

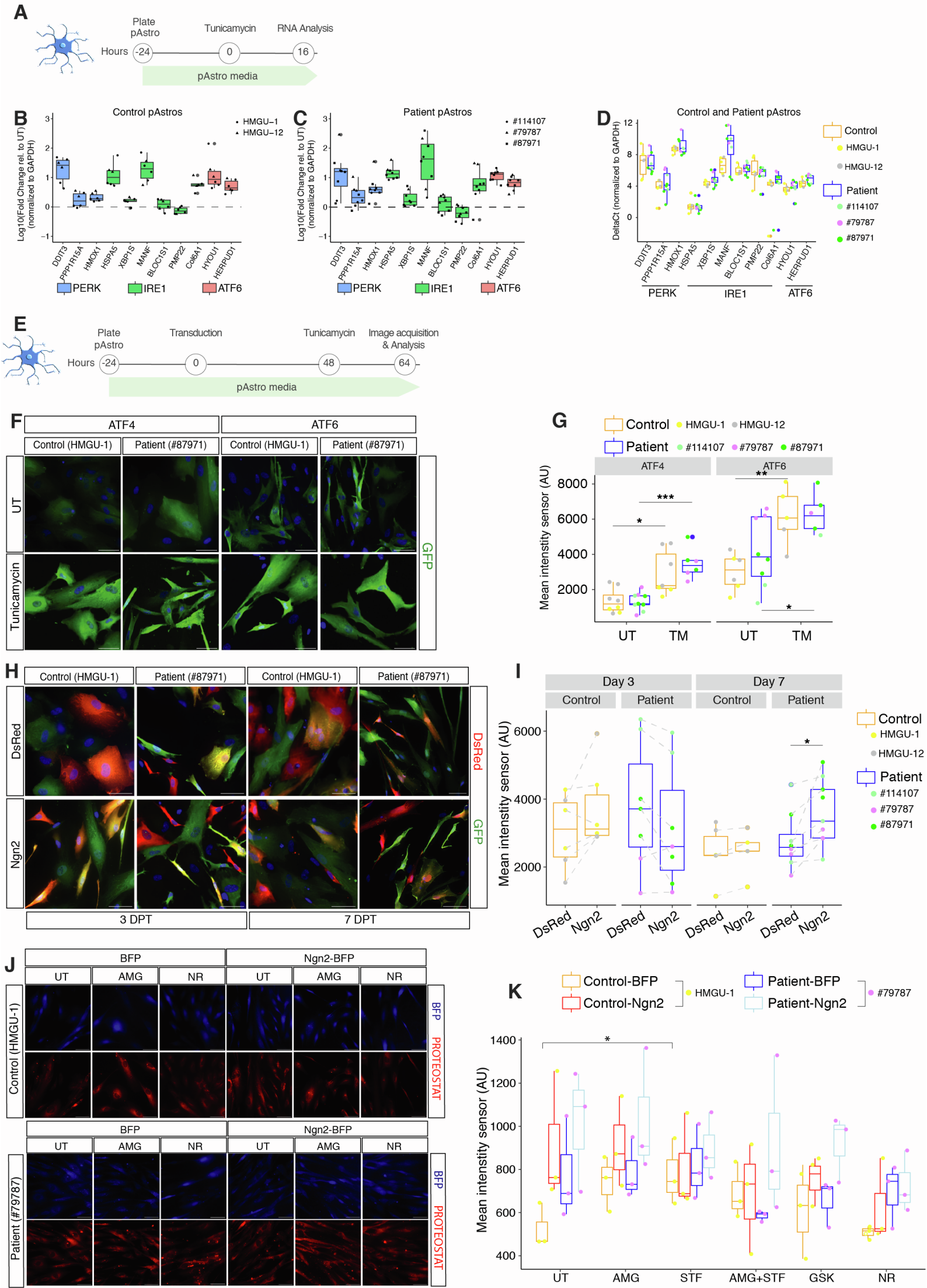
(**B**) Boxplots showing the reprogramming efficiency of control and patient pAstros following the indicated treatments. n=3 independent culture batches per each line. $^{**}p \leq 0.01$.

(**C, D**) Boxplots showing the reprogramming efficiency of control pAstros, patient pAstros and patient pAstros following the expression of atNDB4 and the indicated reprogramming factors in absence (**C**) or presence of Bcl2 (**D**). n=3 independent culture batches per each line. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

(**E**) Micrographs of control and patient fibroblasts transduced with Ngn2 and treated with the indicated small molecules at 20 DPT. Scale bars = 50 µm.

(**F**) Boxplots showing the reprogramming efficiency of control and patient pAstros transduced with Ngn2 and following the indicated treatments. n=3 independent culture batches per each line. $^{*}p \leq 0.05$.

FIGURE S3


A
Plate pAstro
Tunicamycin
RNA Analysis
Hours
-24
0
16
pAstro media
B
Control pAstros
HMGU–1
HMGU–12
Log10(Fold Change rel. to UT) (normalized to GAPDH)
DDIT3
PPP1R15A
HMOX1
HSPA5
XBP1S
MANF
BLOC1S1
PMP22
Col6A1
HYOU1
HERPUD1
PERK
IRE1
ATF6
C
Patient pAstros
#114107
#79787
#87971
D
Control and Patient pAstros
DeltaCt (normalized to GAPDH)
Control
HMGU-1
HMGU-12
Patient
#114107
#79787
#87971
E
Plate pAstro
Transduction
Tunicamycin
Image acquisition & Analysis
Hours
-24
0
48
64
pAstro media
F
ATF4
ATF6
Control (HMGU-1)
Patient (#87971)
UT
Tunicamycin
GFP
G
Control
HMGU-1
HMGU-12
Patient
#114107
#79787
#87971
ATF4
ATF6
Mean intenstity sensor (AU)
UT
TM
H
Control (HMGU-1)
Patient (#87971)
DsRed
Ngn2
GFP
3 DPT
7 DPT
I
Day 3
Day 7
Control
Patient
Mean intenstity sensor (AU)
DsRed
Ngn2
J
BFP
Ngn2-BFP
UT
AMG
NR
Control (HMGU-1)
Patient (#79787)
PROTEOSTAT
K
Control-BFP
Control-Ngn2
Patient-BFP
Patient-Ngn2
HMGU-1
#79787
Mean intenstity sensor (AU)
UT
AMG
STF
AMG+STF
GSK
NR

Supplementary Fig. S3 (related to Fig. 3): UPR activation and proteostasis during direct neuronal reprogramming of pAstros

(**A**) Experimental design.

(**B, C**) Boxplots depicting expression of UPR target genes in untreated control (**B**) patient (**C**) pAstros following the induction of ER stress with tunicamycin treatment. Data is shown as log2 of the fold-change relative to untreated and normalized to GAPDH. Data are shown as median ± IQR. n=2-4 independent culture batches per each line (2 control lines and 3 patient lines).

(**D**) Boxplot depicting the expression of UPR target genes over housekeeping in patient pAstros compared to control pAstros. Data is shown as DeltaCt (Ct_gene/Ct_gapdh) to show the variability across lines. Data are shown as median ± IQR. n=2-4 independent culture batches.

(**E**) Experimental design.

(**F**) Micrographs of control and patient pAstros transduced with ATF4-YFP or ATF6-YFP sensor and treated with 500ng/ml tunicamycin for 16 hours from 2 DPT. Scale bars, 50µm.

(**G**) Boxplots depicting mean fluorescent intensity of ATF4- and ATF6-YFP sensor in control and patient pAstros following tunicamycin treatment. Data are shown as median ± IQR. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. n=3-5 independent culture batches per line (2 control lines and 3 patient lines).

(**H**) Micrographs of control and patient pAstros transduced with ATF6-YFP sensor and DsRed or Ngn2-DsRed at 3 DPT (left) and 7 DPT (right). Scale bars, 50µm.

(**I**) Boxplots depicting mean fluorescent intensity of ATF6-YFP sensor at 3 DPT (left) and 7 DPT (right) in control and patient pAstros. Data are shown as median ± IQR. n=3 independent culture batches per line (2 control lines and 3 patient lines). $*p \leq 0.05$.

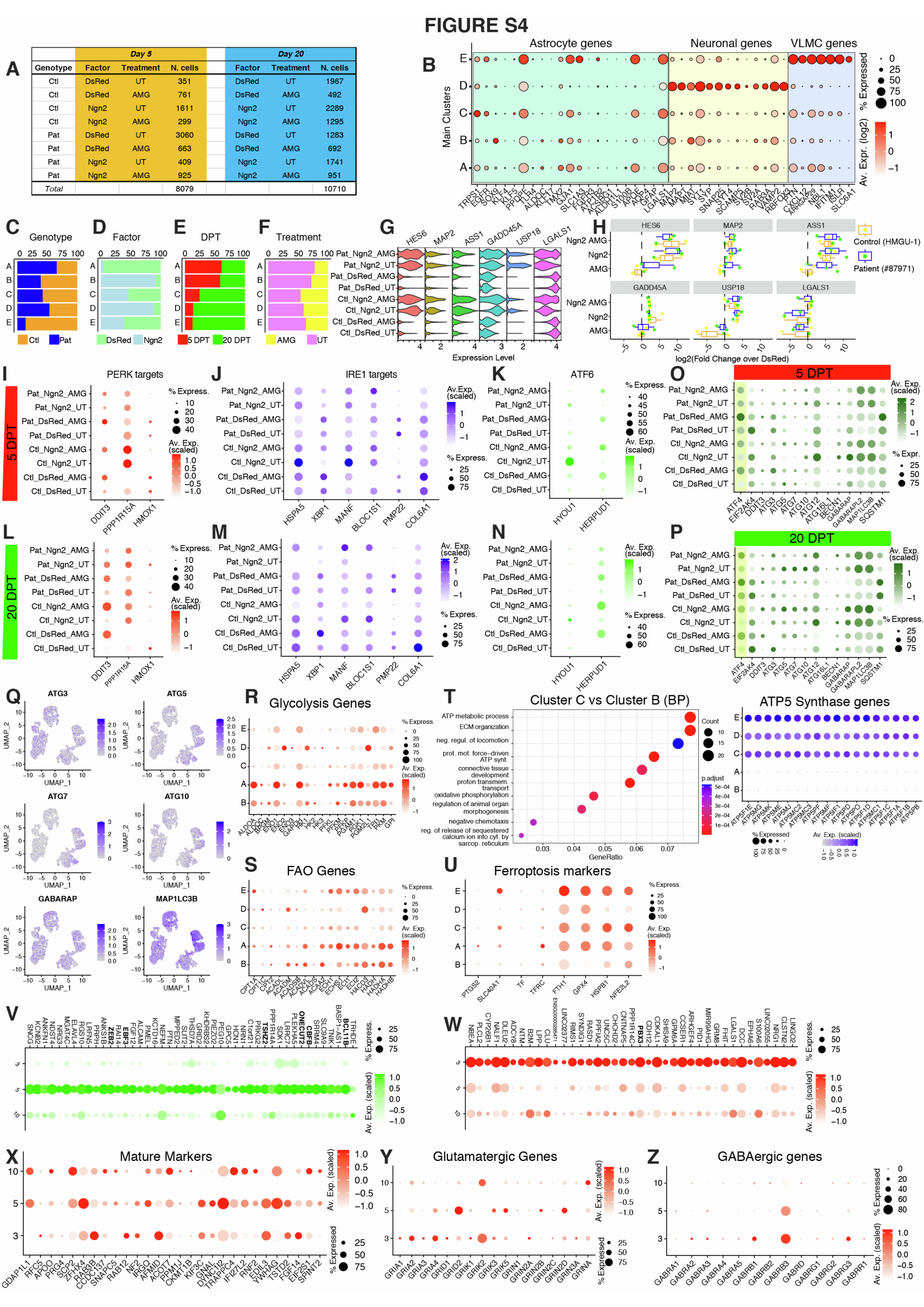
(**J**) Micrographs of control and patient pAstros transduced with BFP or Ngn2-BFP with aggresomes labelled with PROTEOSTAT dye at 5 DPT. Scale bars = 50µm.

(**K**) Boxplots depicting aggresome detection in control and patient pAstros in different experimental conditions at 5 DPT. Data are shown as median ± IQR. $^{*}p \leq 0.05$. n=3 independent culture batches.

FIGURE S4

A

	Day 5			Day 20		
Genotype	Factor	Treatment	N. cells	Factor	Treatment	N. cells
Ctl	DsRed	UT	351	DsRed	UT	1967
Ctl	DsRed	AMG	761	DsRed	AMG	492
Ctl	Ngn2	UT	1611	Ngn2	UT	2289
Ctl	Ngn2	AMG	299	Ngn2	AMG	1295
Pat	DsRed	UT	3060	DsRed	UT	1283
Pat	DsRed	AMG	663	DsRed	AMG	692
Pat	Ngn2	UT	409	Ngn2	UT	1741
Pat	Ngn2	AMG	925	Ngn2	AMG	951
Total			8079			10710



Supplementary Fig. S4 (related to Fig. 4): scRNA-Seq analysis of direct reprogramming of control and patient pAstros with or without AMG treatment

(**A**) Table summarizing the conditions and the number of cells considered for the analysis.

(**B**) Dotplots depicting the scaled expression the indicated genes in different clusters.

(**C-F**) Barplot depicting the cluster composition according to the genotype (**C**)**,** factor (**D**), days post transduction (**E**) and treatment (**F**).

(**G, H**) Violin plot of the expression of selected genes from scRNAseq (**G**) and boxplot showing the log2-fold change in the expression of selected candidate genes via qRT-PCR (**H**).

(**I-N**) Dotplots depicting the scaled expression of known PERK (**I, L**), IRE1 (**J, M**) and ATF6 targets (**K, L**) in different clusters at 5 DPT (**I, K**) or 20 DPT (**L, N**).

(**O, P**) Dotplots depicting the scaled expression of known ISR targets at 5 DPT (**O**) or 20 DPT (**P**).

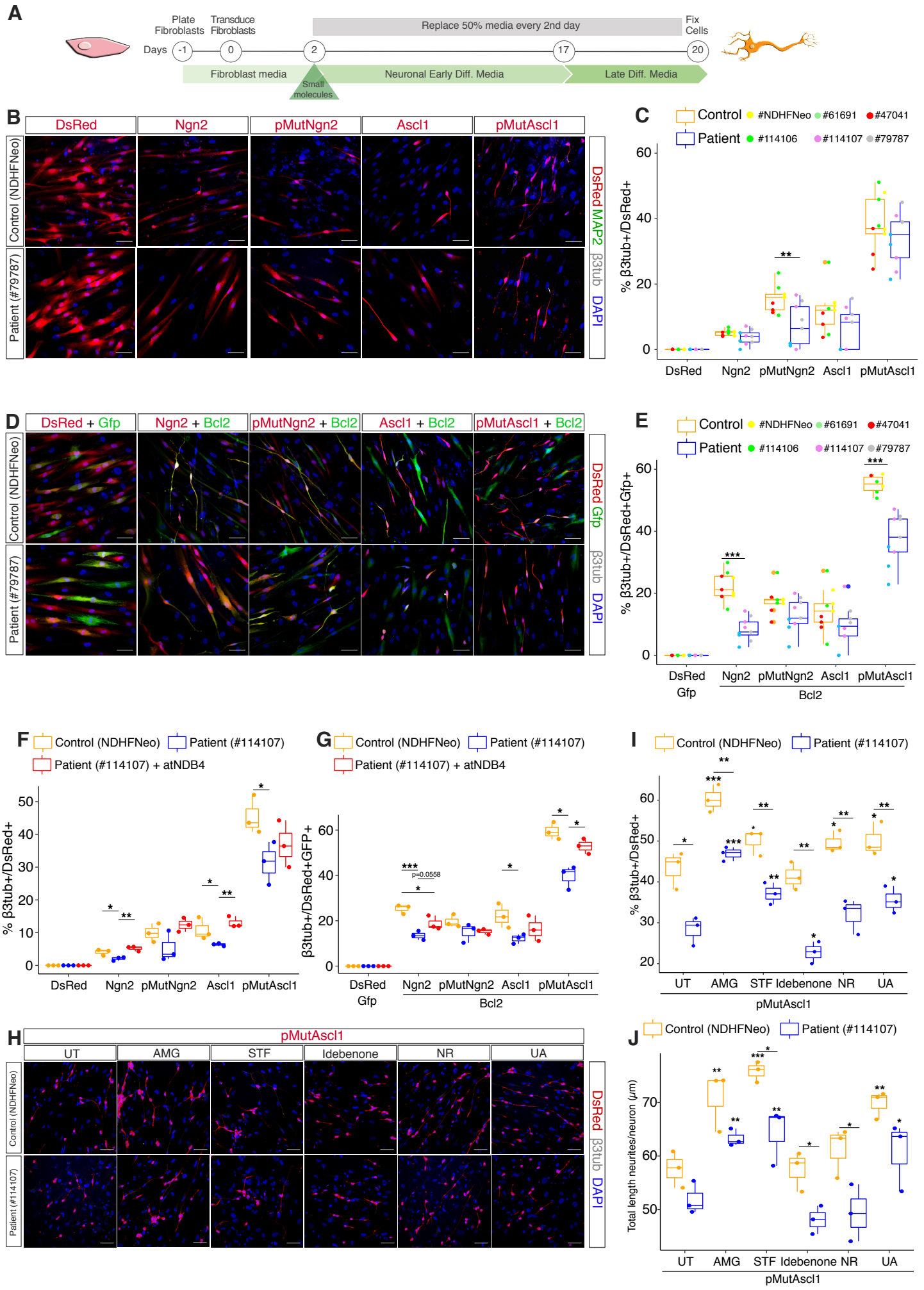
(**Q**) Expression of ISR targets projected into UMAP.

(**R-U**) Dotplots depicting the scaled expression of genes associated to glycolysis (**R**), FAO (**S**), ATP5 synthase (**T, right**) and ferroptosis (**U**). In (**T, left**) top 10 GO (BP) from the comparison of cluster C vs cluster B.

(**V, W**) Dotplots depicting the scaled expression of genes significantly upregulated in cluster 5 (**V**) or in cluster 3 (**W**). Transcription factors are highlighted in bold.

(X-Z) Dotplots depicting the scaled expression of mature neuronal marker genes (**X**), glutamatergic genes (**Y**) and GABAergic genes (**Z**).

FIGURE S5


A
Plate Fibroblasts
Transduce Fibroblasts
Replace 50% media every 2nd day
Fix Cells
Days -1 0 2 17 20
Fibroblast media
Small molecules
Neuronal Early Diff. Media
Late Diff. Media
B
DsRed
Ngn2
pMutNgn2
Ascl1
pMutAscl1
Control (NDHFNeo)
Patient (#79787)
DsRed MAP2 β3tub DAPI
C
Control #NDHFNeo #61691 #47041
Patient #114106 #114107 #79787
% β3tub+/DsRed+
DsRed Ngn2 pMutNgn2 Ascl1 pMutAscl1
D
DsRed + Gfp
Ngn2 + Bcl2
pMutNgn2 + Bcl2
Ascl1 + Bcl2
pMutAscl1 + Bcl2
DsRed Gfp β3tub DAPI
E
% β3tub+/DsRed+Gfp+
DsRed Gfp
Ngn2 pMutNgn2 Ascl1 pMutAscl1
Bcl2
F
Control (NDHFNeo)
Patient (#114107)
Patient (#114107) + atNDB4
% β3tub+/DsRed+
G
β3tub+/DsRed+GFP+
p=0.0558
I
% β3tub+/DsRed+
UT AMG STF Idebenone NR UA
pMutAscl1
H
pMutAscl1
UT AMG STF Idebenone NR UA
Patient (#114107)
J
Total length neurites/neuron (μm)

Supplementary Fig. S5 (related to Figure 2): Impaired neuronal reprogramming of NDUFS4-patient fibroblasts and pharmacological rescue

(**A**) Experimental design.

(**B, D**) Micrographs depicting control (NDHFNeo) and patient (#79787) fibroblasts transduced with the indicated transcription factors (**B**: single factor; **D**: two factors) at 20 DPT. Scale bars = 50 μm.

(**C, E**) Boxplots of the reprogramming efficiency of control and patient fibroblasts following single (**C**) or double (**E**) transcription factors transduction. Data are shown as median ± IQR. n=3 independent culture batches per each cell line. $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

(**F, G**) Boxplots showing the reprogramming efficiency of control, patient fibroblasts and patient fibroblasts stably expressing atNDB4, transduced with he indicated reprogramming factors in absence (**F**) or presence of Bcl2 (**G**). Asterisks with a bar depict the significance between control and patient sample. Data are shown as median ± IQR. $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. n=3 independent culture batches.

(**H**) Micrographs depicting control and patient fibroblasts transduced with pMutAscl1 alone or in combination with AMG-PERK 44 (AMG), STF-083010 (STF), Idebenone, Nicotinamide Riboside (NR) or Urolithin A (UA) treatment at 20 DPT. Scale bars = 50 μm.

(**I, J**) Boxplots of reprogramming efficiency (**I**) and neurite process length (**J**) of control and patient fibroblasts treated with the indicated small molecules. Asterisks indicate the significance over the corresponding untreated sample; asterisks above a bar depict the significance between control and patient sample. Data are shown as median ± IQR. $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$. n=3 independent culture batches.