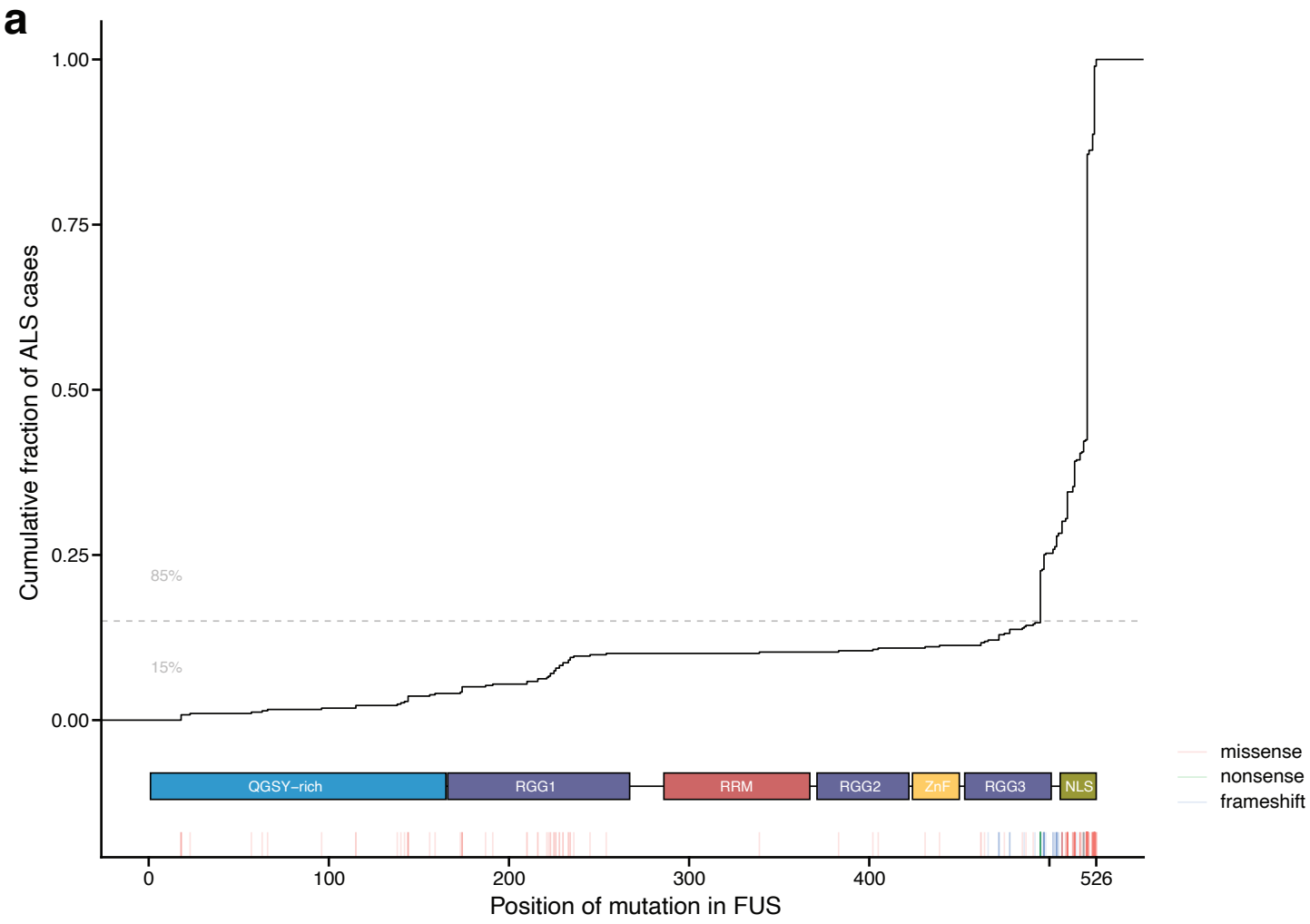
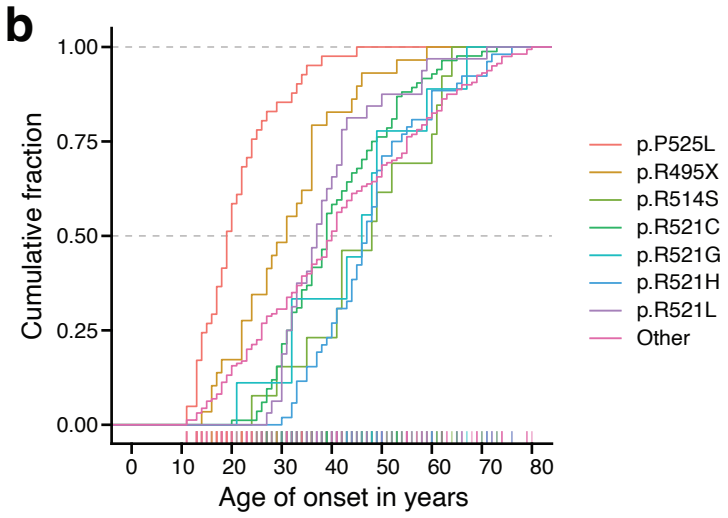
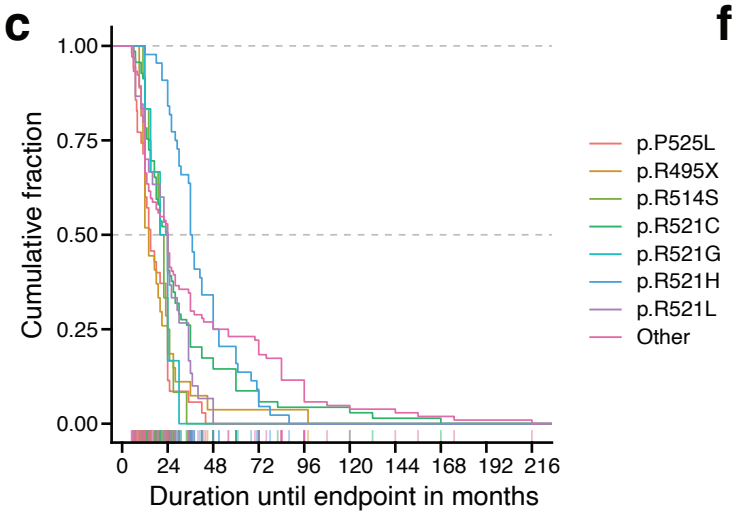
Supplementary Figures

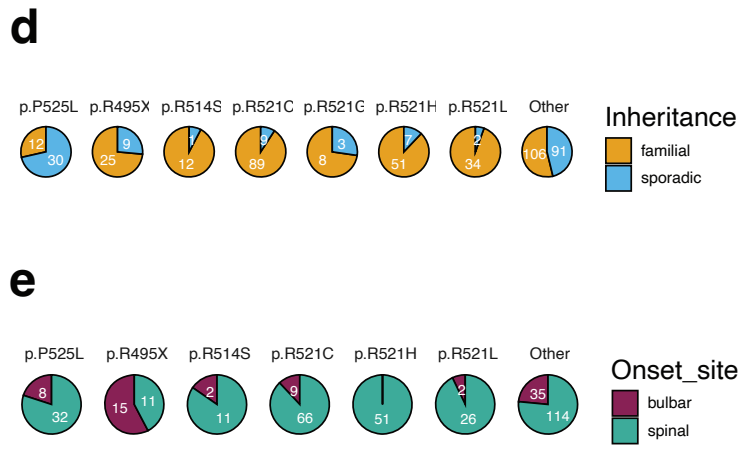
Single-cell RNA sequencing reveals early mitochondrial dysfunction unique to motor neurons shared across *FUS*- and *TARDBP*-ALS

Christoph Schweingruber[1,2], Jik Nijssen[2], Jonas Mechtersheimer[3], Stefan Reber[3], Melanie Leboeuf[1,2], Niamh O'Brien[3], Irene Mei[1], Erin Hedges[3], Michaela Keuper[4], Julio Aguila Benitez[2], Vlad Radoi[1], Martin Jastroch[4], Marc-David Ruepp[3*], Eva Hedlund[1,2*]

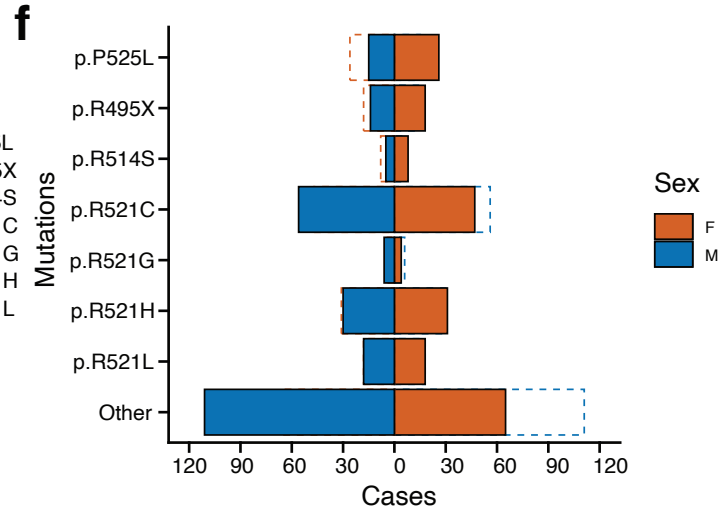

a
Cumulative fraction of ALS cases
1.00
0.75
0.50
0.25
0.00
85%
15%
QGSY−rich
RGG1
RRM
RGG2
ZnF
RGG3
NLS
missense
nonsense
frameshift
0
100
200
300
400
526
Position of mutation in FUS


b
Cumulative fraction
1.00
0.75
0.50
0.25
0.00
0
10
20
30
40
50
60
70
80
Age of onset in years
p.P525L
p.R495X
p.R514S
p.R521C
p.R521G
p.R521H
p.R521L
Other


c
Cumulative fraction
1.00
0.75
0.50
0.25
0.00
0
24
48
72
96
120
144
168
192
216
Duration until endpoint in months
p.P525L
p.R495X
p.R514S
p.R521C
p.R521G
p.R521H
p.R521L
Other


d
p.P525L
p.R495X
p.R514S
p.R521C
p.R521G
p.R521H
p.R521L
Other
12
30
25
9
12
89
8
3
51
7
34
2
106
91
Inheritance
familial
sporadic

e
p.P525L
p.R495X
p.R514S
p.R521C
p.R521H
p.R521L
Other
8
32
15
11
2
11
9
66
51
2
26
35
114
Onset_site
bulbar
spinal


f
Mutations
p.P525L
p.R495X
p.R514S
p.R521C
p.R521G
p.R521H
p.R521L
Other
120
90
60
30
0
30
60
90
120
Cases
Sex
F
M

Supplementary Figure 1. Meta-analysis of previously reported ALS cases with FUS mutations. Clinical features from 502 individual cases with *FUS*-ALS were collected and aggregated from 115 articles or case reports published between 2009 and 2022. **a**, The ALS cases with FUS mutations were plotted as empirical cumulative density over the FUS protein. The c-terminal mutations account for approximately five sixth of the mutations reported in the dataset. **b-f**, Clinical features for *FUS* mutations with more than 10 cases in the dataset. The group 'Other' contains all the remaining mutations in the dataset. **b**, Age at onset in years is significantly different between FUS mutations (log-rank test, $\chi^2 = 175$, df = 7, $p < 2e\text{-}16$). **c**, Duration to endpoint (defined as respiratory failure or death) in months is significantly different between FUS mutations (log-rank test, $\chi^2 = 69.4$, df = 7, $p < 2e\text{-}12$). **d**, Inheritance of ALS (as familial or sporadic). **e**, The site of onset. **f**, Sex disparity.[1–115]

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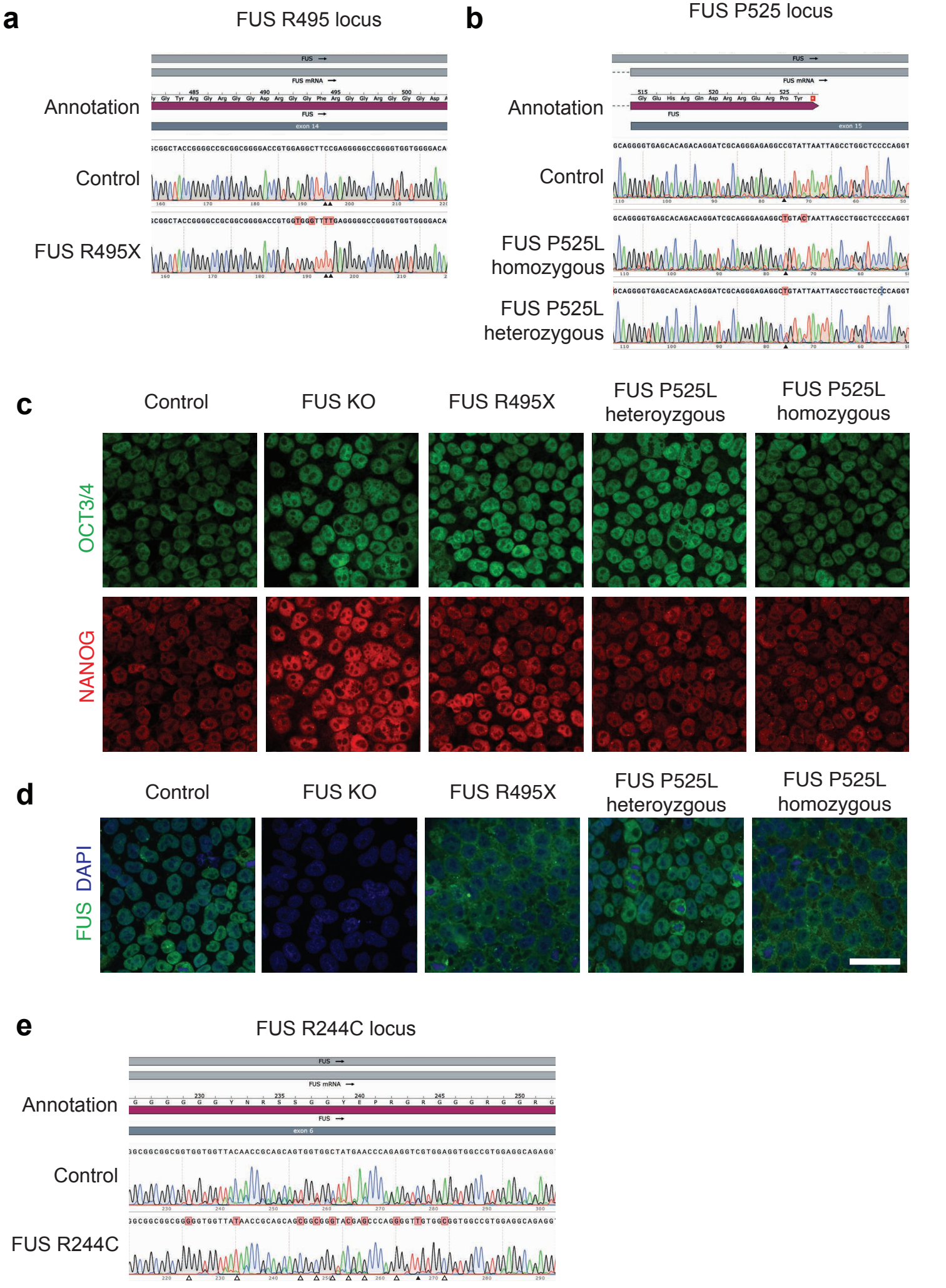

Supplementary Figure 2. Characterization of human iPSC lines with FUS mutations. Following genome editing of the DF6-9-9T.B control line, the editing sites were interrogated by dye terminator sequencing. **a**, Chromograms show the successful introduction of the R495X mutation in exon 14 of *FUS* in contrast to control cells. **b**, Chromograms show the introduction of the P525L mutation in exon 15 of *FUS* in a heterozygous and homozygous line. The editing of the FUS KO line was described previously [116,117]. **c**, Pluripotency of the genome-edited human iPSC lines was assessed with immunostainings with the stem cell markers OCT3/4 and NANOG. **d**, FUS expression and localization in the human iPSC lines was assessed by immunofluorescence with an antibody against N-terminal FUS and nuclear counterstaining with DAPI. **e**, Chromograms show the introduction of the R244C mutation in exon 6 of *FUS*. The white arrows indicate silent point mutations preventing re-editing of the locus whereas the black arrow indicates the target point mutation.

Supplementary Figure 3

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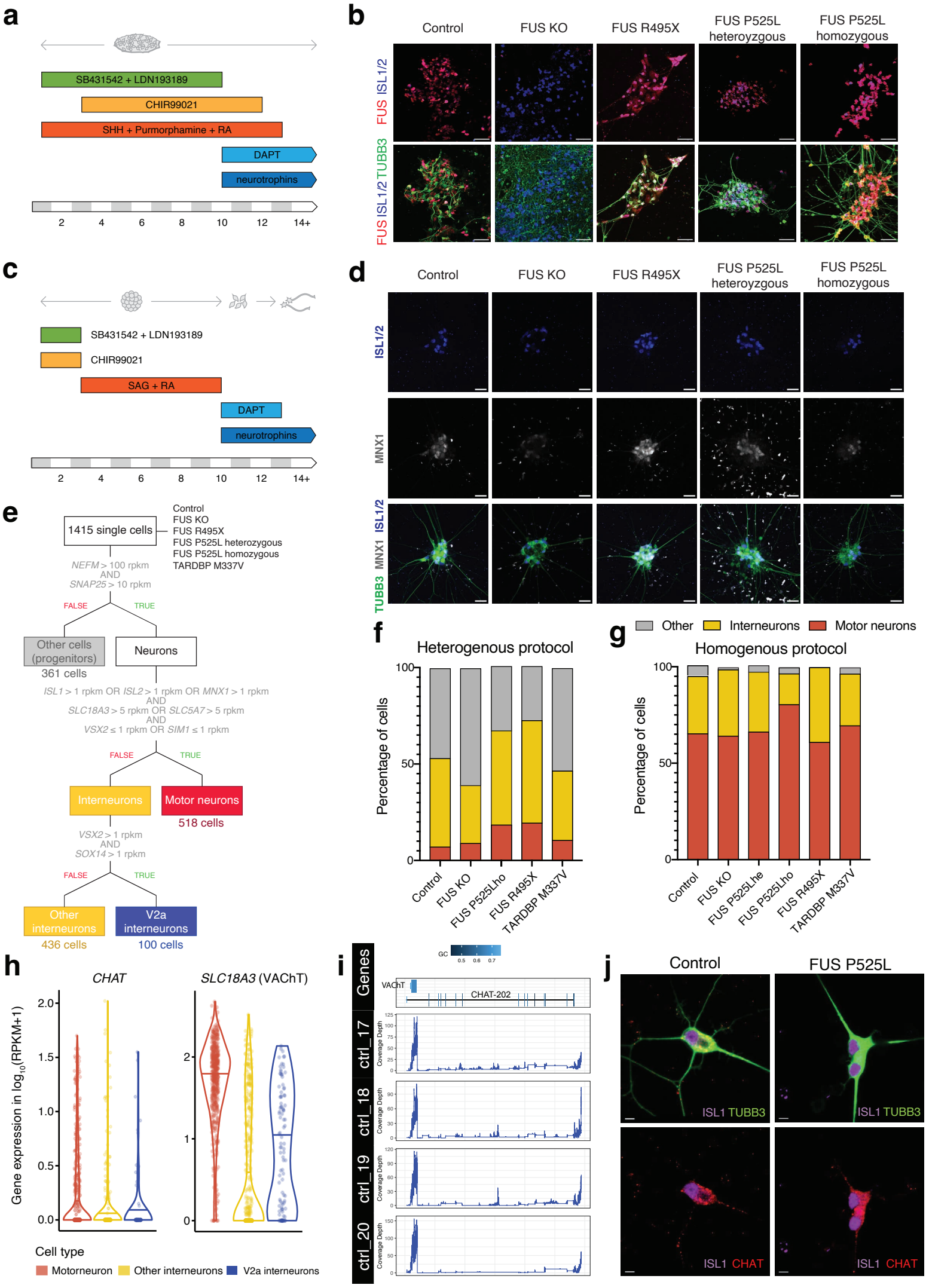
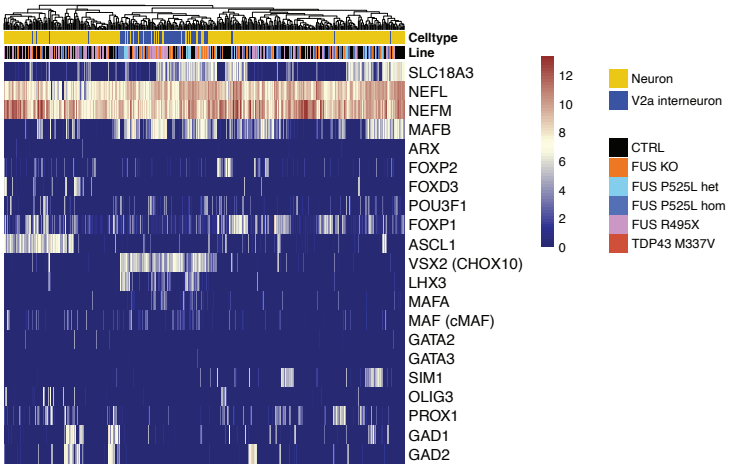
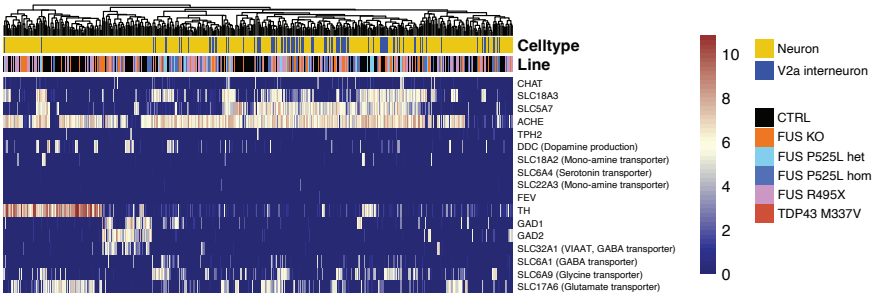


Supplementary Figure 3. Differentiation of human iPSC to diverse spinal neurons. a, Schematic of the differentiation protocol that yields a heterogenous population of spinal neurons including motor neurons. **b**, Expression of the motor neuron markers ISL1/2 and TUBB3 and FUS expression in the heterogenous protocol was assessed by confocal immunofluorescence microscopy. The scale bars measure 25 µm. **c**, Schematic of the differentiation protocol that yield a homogenous population of mostly spinal motor neurons. **d**, Expression of the motor neuron markers ISL1/2, MNX1 (Hb9) and TUBB3 in the homogenous protocol was assessed by confocal immunofluorescence microscopy. The scalebars measure 25 µm. **e,** The decision tree used for the cell type classification in our single cell RNA-Seq analysis. The specific expression cut-offs at each branch point are depicted. **f, g**, Following single cell RNA-Seq and cell-type classification, the proportions of motor neurons and interneurons and other cells (mostly neural progenitors) were calculated for the heterogenous **e**, and homogenous **f**, protocol, respectively. **h**, Expression of cholinergic markers *CHAT* and *SLC18A3* (VAChT) across cell types. **i**, Gene coverage plots for the nested CHAT-SLC18A3 genetic locus in four individual cells. **j**, Expression of the cholinergic marker CHAT in motor neurons ($ISL1^+$, $Tuj1^+$) specified with the homogenous protocol from control iPSC.

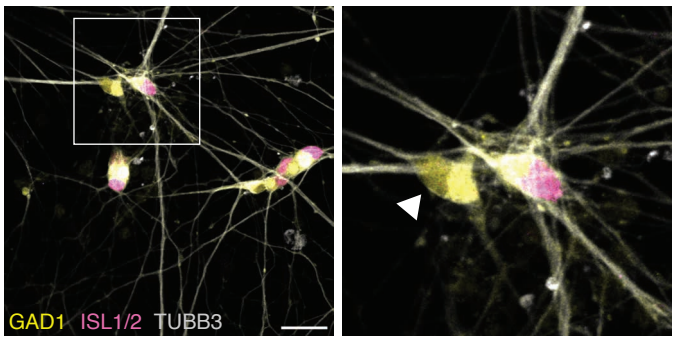
a Neuronal marker genes



b Neurotransmitter biosynthesis

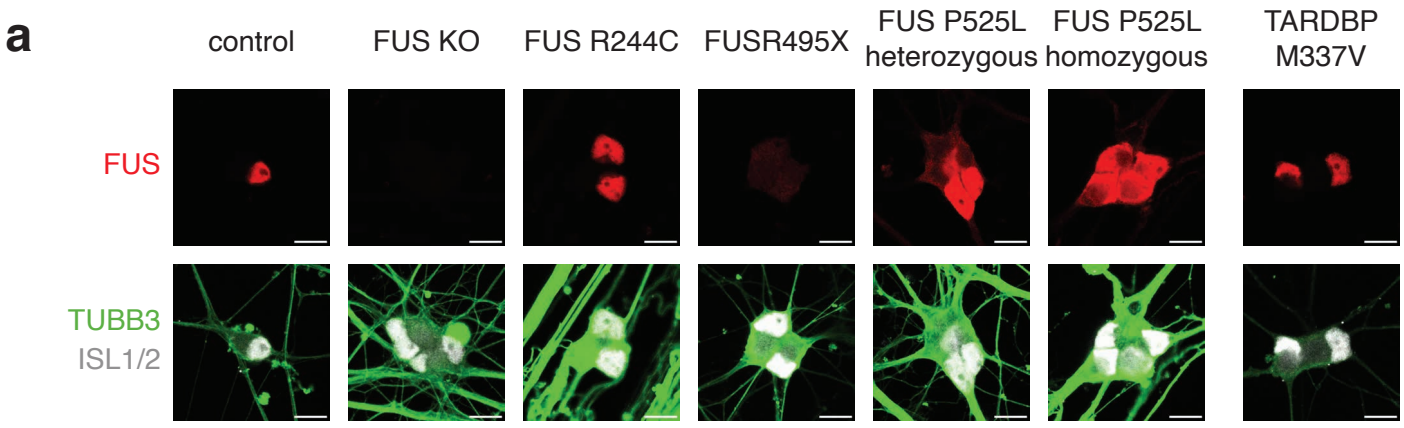


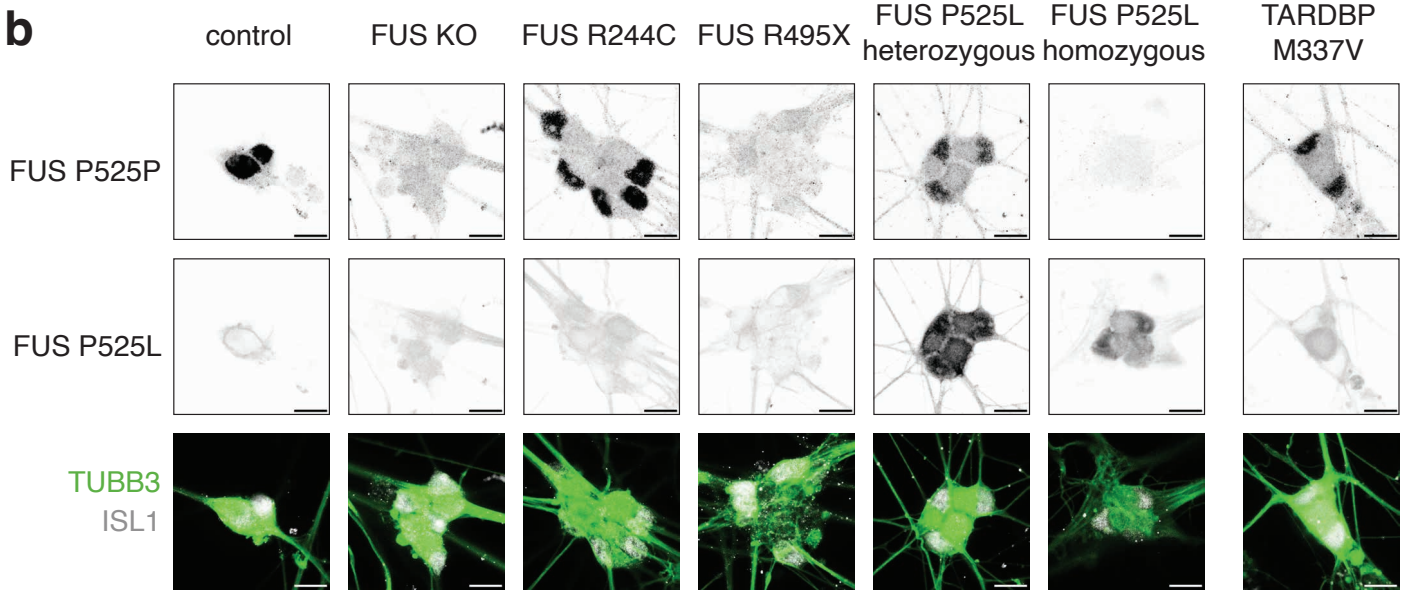
c

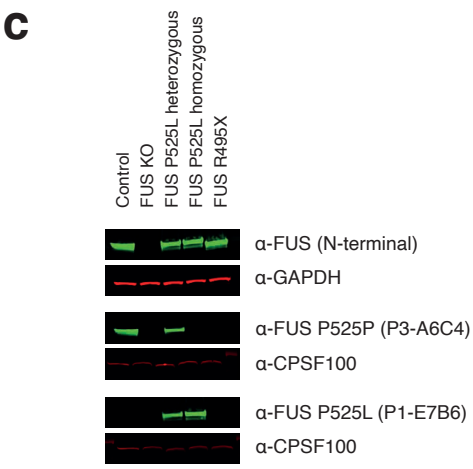


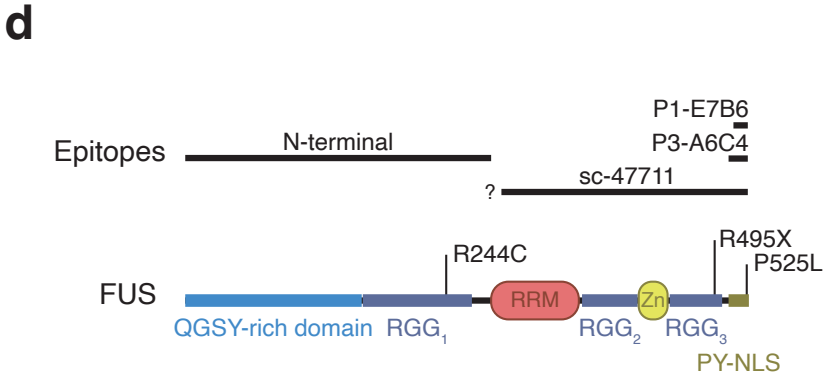
Supplementary Figure 4. Several interneuron populations are present in the dataset.

a, Heat map of the expression of selected markers in the other neurons and V2a interneurons. Hierarchical clustering groups the *VSX2*-expressing V2a interneurons. **b,** Heat map of neurotransmitter biogenesis and transporter gene expression in other neurons and V2a interneurons. Hierarchical clustering separates subgroups expressing *TH* (initiating the adrenergic and derivative neurotransmitter synthesis) and GABA-ergic neurons (*GAD1*, *GAD2*, *SLC32A1*, *SLC6A1*) among others. **c,** The presence of GABAergic interneurons (TUBB3+, ISLET1/2-, GAD1+, white arrow) among the motor neurons (TUBB3+, ISLET1/2+, GAD1+) was assessed by confocal immunofluorescence microscopy. The scale bars measure 30 µm.


a
control
FUS KO
FUS R244C
FUSR495X
FUS P525L heterozygous
FUS P525L homozygous
TARDBP M337V
FUS
TUBB3
ISL1/2


b
control
FUS KO
FUS R244C
FUS R495X
FUS P525L heterozygous
FUS P525L homozygous
TARDBP M337V
FUS P525P
FUS P525L
TUBB3
ISL1


c
Control
FUS KO
FUS P525L heterozygous
FUS P525L homozygous
FUS R495X
α-FUS (N-terminal)
α-GAPDH
α-FUS P525P (P3-A6C4)
α-CPSF100
α-FUS P525L (P1-E7B6)
α-CPSF100


d
Epitopes
N-terminal
P1-E7B6
P3-A6C4
sc-47711
?
FUS
R244C
R495X
P525L
RRM
Zn
QGSY-rich domain
RGG1
RGG2
RGG3
PY-NLS

Supplementary Figure 5. The intracellular localization of FUS is disturbed in ALS motor neurons. a, Confocal fluorescence microscopy with immunostaining of C-terminal FUS in motor neurons (TUBB3+, ISL1/2+) at day 28 reveals cytoplasmic accumulation of FUS P525L and ablation in the FUS KO line. Scale bars are 10 µm. **b**, Interrogation with antibodies specific to FUS P525 epitopes allow us to separate the contribution of wild-type (P525P) and mutant (P525L) protein to the mis-localized pool of FUS in motor neurons at day 28. Scale bars are 10 µm. **c**, The specificity of the antibodies against the P525 epitope was assessed by western blot with lysates from the FUS mutant iPSC lines. **d**, Epitope map of the FUS antibodies used in this study including the FUS domain architecture as reference.

Supplementary Figure 6

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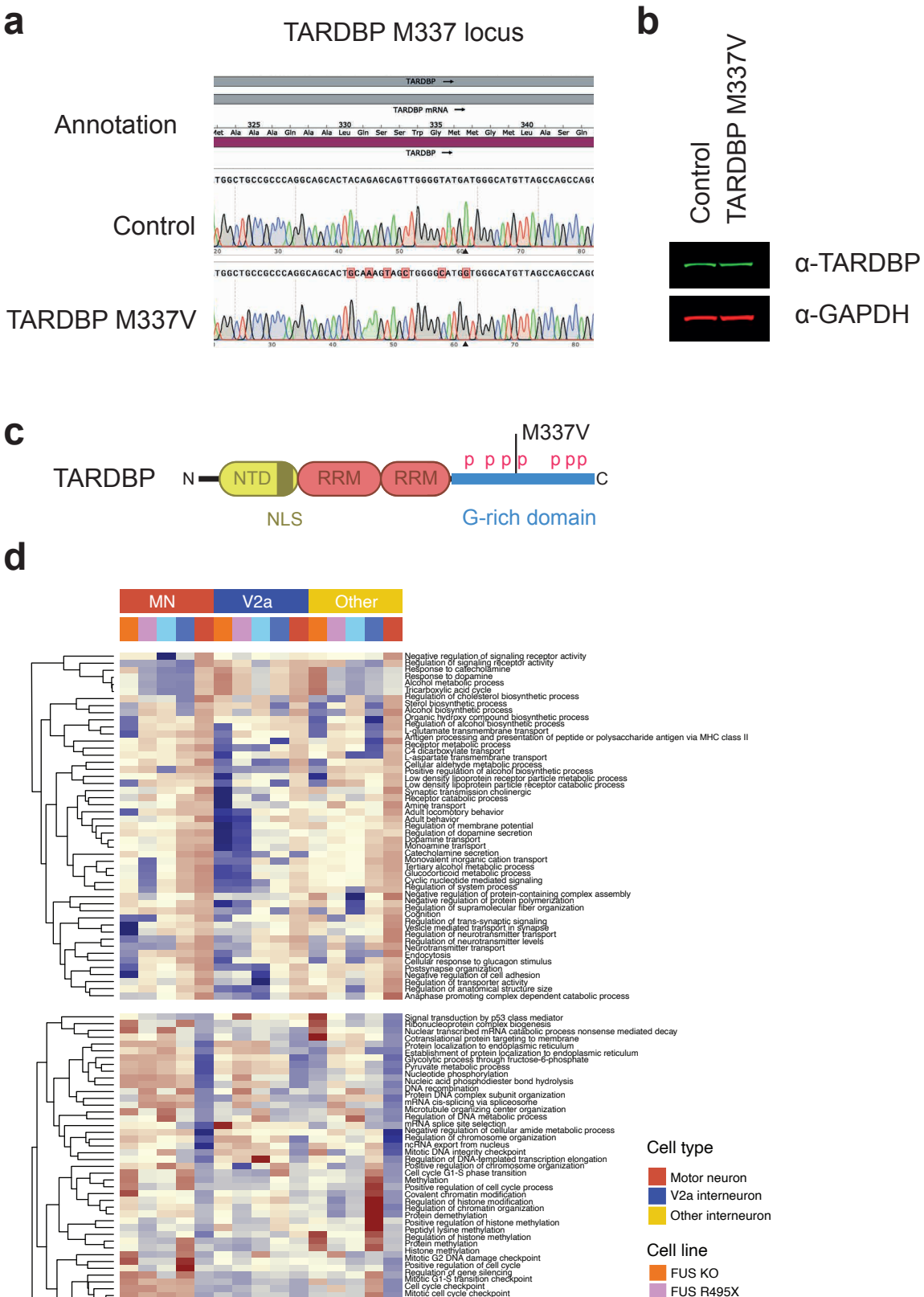

Supplementary Figure 6. Characterization of the iPSC line with the TARDBP M337V mutation. Following genome editing of the DF6-9-9T.B control line, the editing site was interrogated by dye terminator sequencing. **a,** Chromogram of the TARDBP M337 locus show the successful editing in the human iPSC line. **b,** Expression of TARDBP was assessed by western blot in lysates from control and mutant human iPSC lines. **c,** Domain architecture of TARDBP indicating the phosphorylation sites and location of the M337V mutation. **d**, Heatmaps display the normalized enrichment scores from GSEA of the top 25 up- and downregulated biological processes uniquely regulated in TARDBP M337V motor neurons. Motor neurons are compared to V2a or other interneurons to show specific or stronger regulation of these pathways in motor neurons.

Supplementary Figure 7

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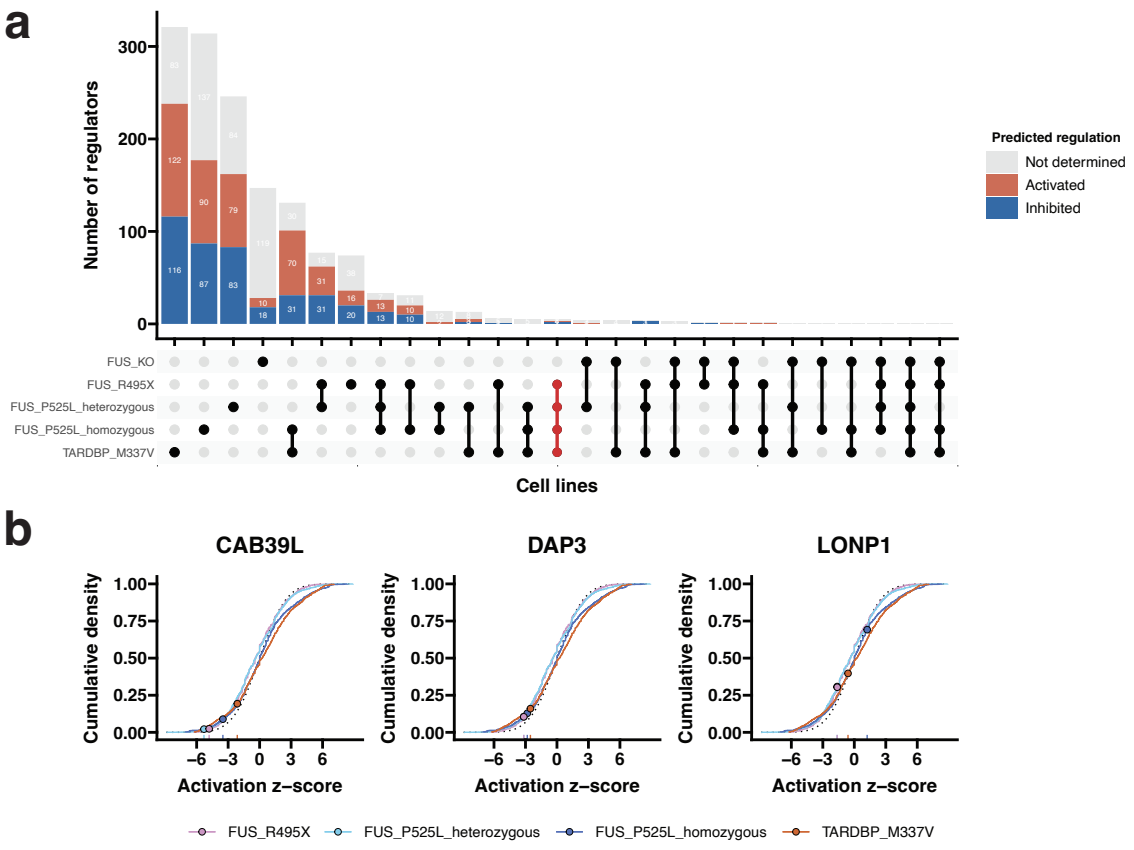

Supplementary Figure 7. Upstream regulators of shared dysregulation in ALS lines.

Upstream regulators that could explain the expression changes in the individual ALS-mutant motor neurons compared to the control were analyzed in the IPA tool set. **a,** The upset plot shows the intersection of upstream regulators with a network bias-corrected *p*-value < 0.001 separated according to their predicted regulation. The upstream regulator set that is shared across ALS lines is highlighted in red. **b,** The activation *z*-score for the protein-coding upstream regulators in the shared set are shown against the set of all upstream regulators in cumulative density plots. While a negative activation *z*-score indicates inhibition of the regulator, a positive score indicates its activation. CAB39L, DAP3, and LONP1 are all mitochondrial genes.

Supplementary Figure 8

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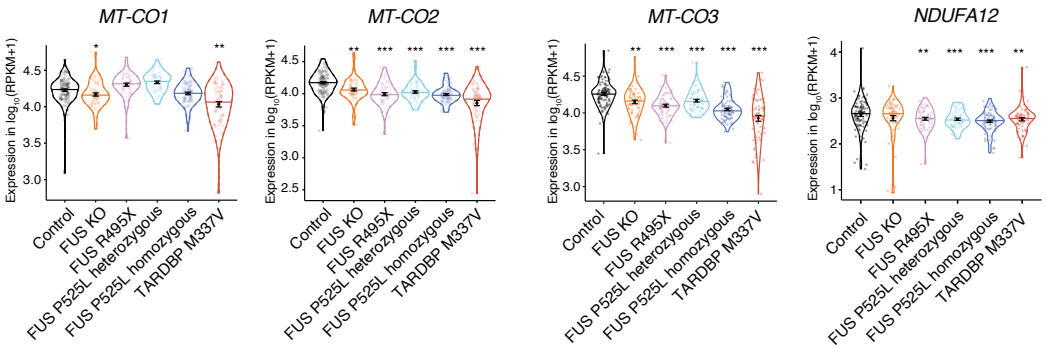
a



Supplementary Figure 8. Expression of selected mitochondrial encoded genes.

Violin plots showing normalized expression values (RPKM) of MT-CO1, MT-CO2, MT-CO3, and NDUFA12 in motor neurons generated with the homogeneous protocol. Error bars show mean ± SEM. The significance levels of the p-values are * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. control, derived from DESeq2 two-sided differential gene expression analysis.

Supplementary Figure 9

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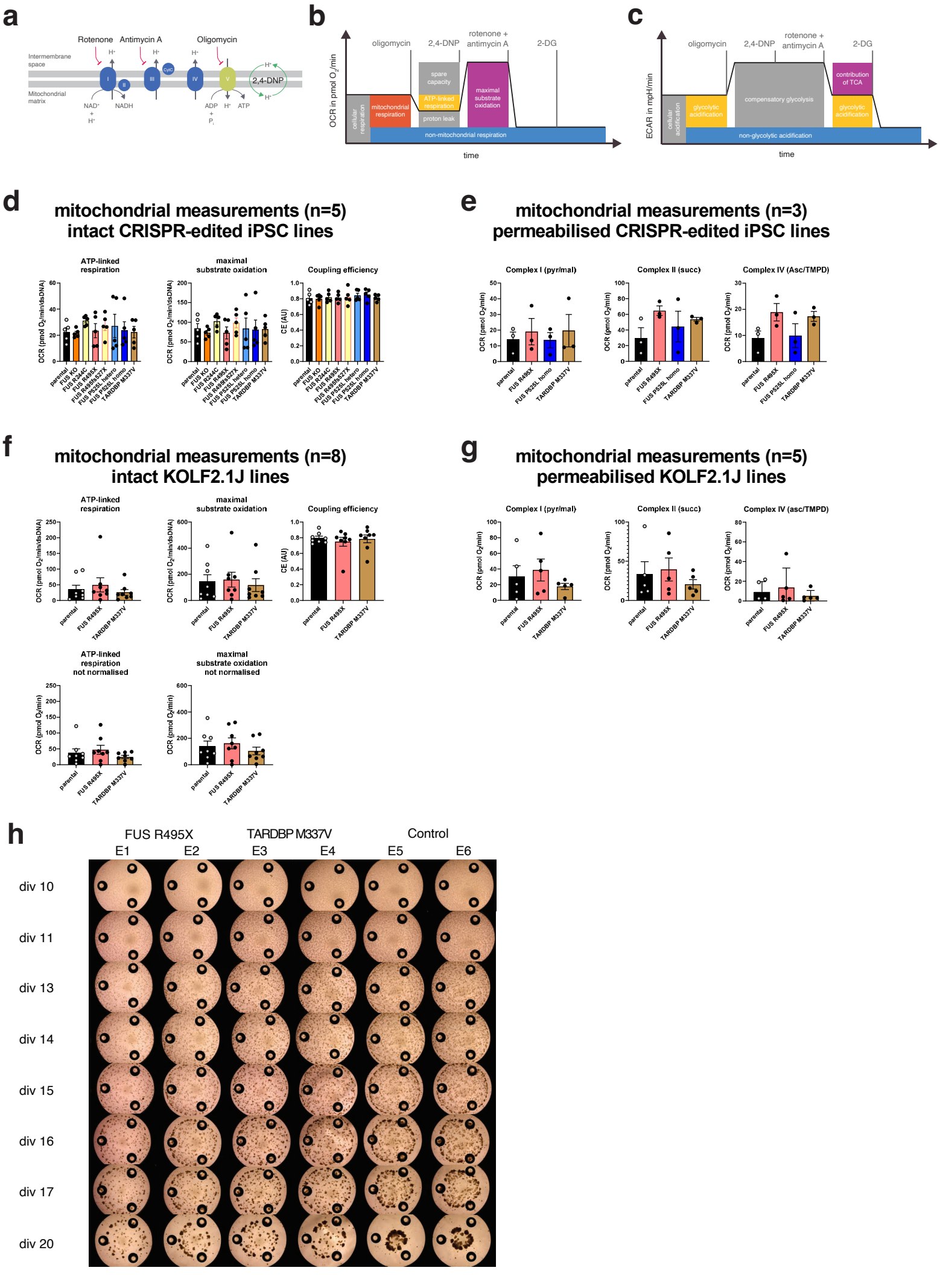

Supplementary Figure 9. Bioenergetic profiling of mitochondria in motor neurons. Bioenergetic profiling was conducted in motor neurons from the homogenous protocol at day 21. **a**, Specific mitochondrial inhibitors (oligo: oligomycin, R: rotenone, AA: antimycin A) and the uncoupler 2,4-Dinitrophenol (2,4-DNP), as depicted in the graph, and D-glucose metabolism inhibitor 2-deoxyglucose (2-DG), were used to analyzed oxygen consumption (OCR) and extracellular acidification (ECAR) using a XF96 extracellular flux analyzer as described in the Methods section. **b-c,** Partitioning of OCR (**b**) and ECAR (**c**) into different functional modules as depicted in the scheme (TCA: tricarboxylic acid cycle). **d-e,** Key metabolic parameters in intact and permabilized motor neurons derived from the DF-6-9-9T.B iPSC lines used in the single cell RNA sequencing. **f-g,** Key metabolic parameters in intact and permabilized motor neurons derived from KOLF2.1J iPSC lines. Data are the mean ± SEM of three to eight independent differentiations as indicated. * $p < 0.05$, + $p < 0.001$, # $p < 0.0001$ vs. control, One-way ANOVA (posthoc: Dunnett's). **h,** Representative images from motor neuron cultures in the KOLF2.1J background leading up to the bioenergetic profiling.

Supplementary References

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