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Supplemental Information

Reactive Astrocytes Promote ALS-like Degeneration and Intracellular Protein Aggregation in Human Motor Neurons by Disrupting Autophagy through TGF-β1

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Supplementary Figures and Legends

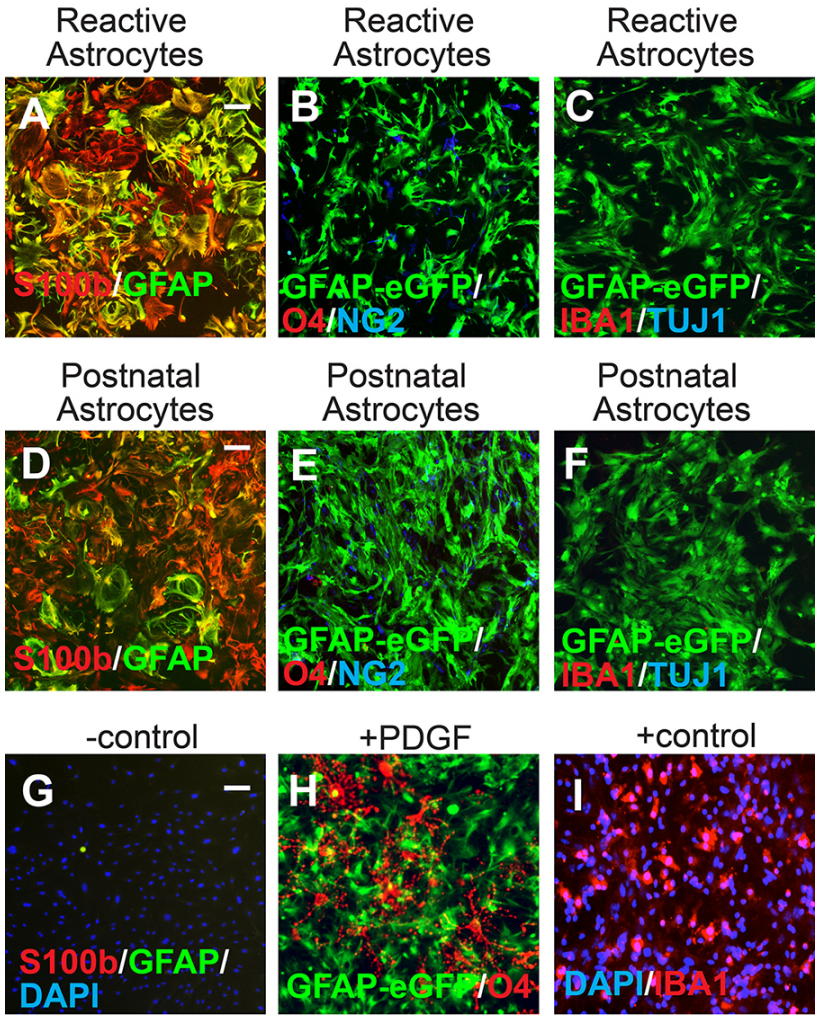
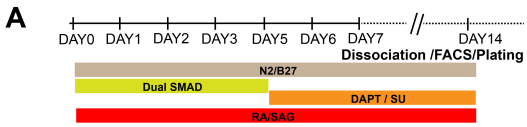
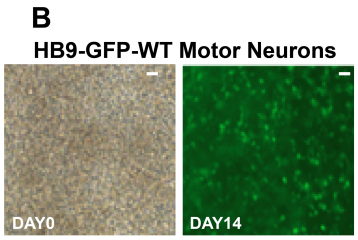


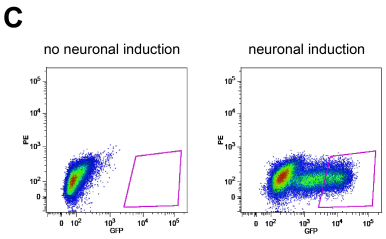
Figure S1: Immunohistochemical analysis of mutant astrocytes (associated with Figure 1).

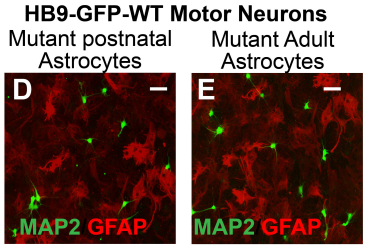
Immunohistochemistry showed that astrocyte cultures obtained from adult and postnatal mouse spinal cord are highly enriched for $GFAP^{+}S100b^{+}$astrocytes (**A, D**) and devoid of $TUJ1^{+}$ neurons, $O4^{+}$ oligodendrocytes, and $IBA1^{+}$ microglia (**B, C, E, G**). A small number of $NG2^{+}$ cells are present in the cultures (**B, E**). A mixed

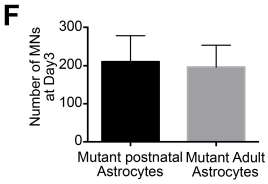
culture of embryonic mouse brain served as positive controls for O4 and IBA1 antibody staining (**H, I**).

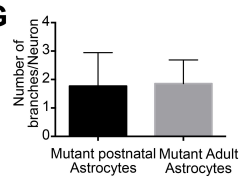

A
DAY0 DAY1 DAY2 DAY3 DAY5 DAY6 DAY7 DAY14
Dissociation /FACS/Plating
N2/B27
Dual SMAD
DAPT / SU
RA/SAG

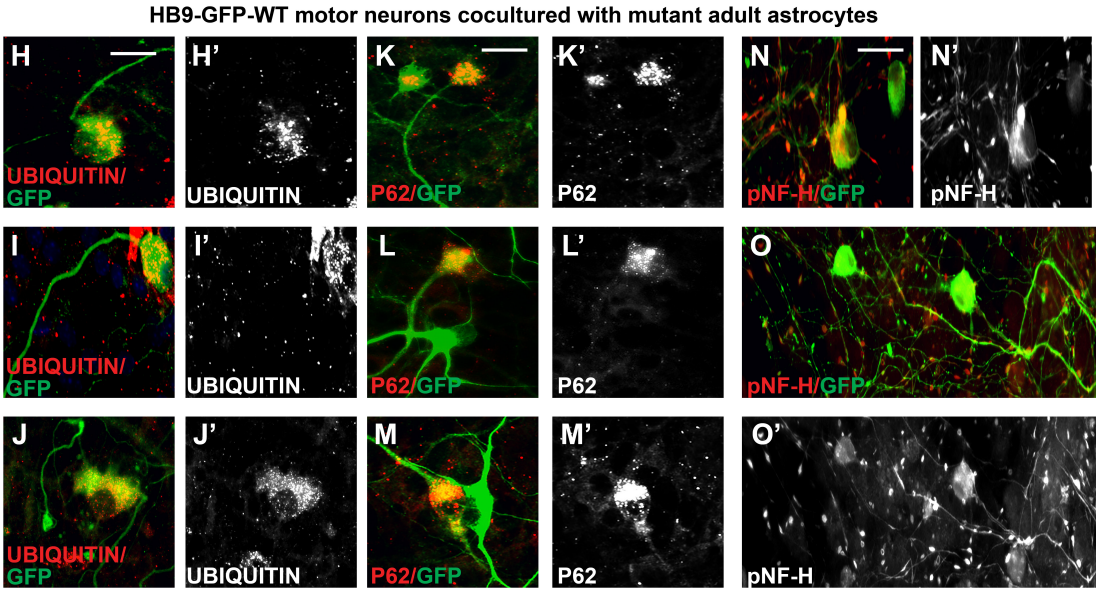

B
HB9-GFP-WT Motor Neurons
DAY0
DAY14


C
no neuronal induction
neuronal induction
PE
GFP


HB9-GFP-WT Motor Neurons
Mutant postnatal Astrocytes
Mutant Adult Astrocytes
D
E
MAP2 GFAP
MAP2 GFAP


F
Number of MNs at Day3
300
200
100
0
Mutant postnatal Astrocytes
Mutant Adult Astrocytes


G
Number of branches/Neuron
4
3
2
1
0
Mutant postnatal Astrocytes
Mutant Adult Astrocytes


HB9-GFP-WT motor neurons cocultured with mutant adult astrocytes
H
UBIQUITIN/ GFP
H'
UBIQUITIN
K
P62/GFP
K'
P62
N
pNF-H/GFP
N'
pNF-H
I
UBIQUITIN/ GFP
I'
UBIQUITIN
L
P62/GFP
L'
P62
O
pNF-H/GFP
J
UBIQUITIN/ GFP
J'
UBIQUITIN
M
P62/GFP
M'
P62
O'
pNF-H

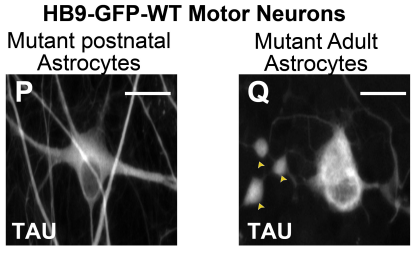
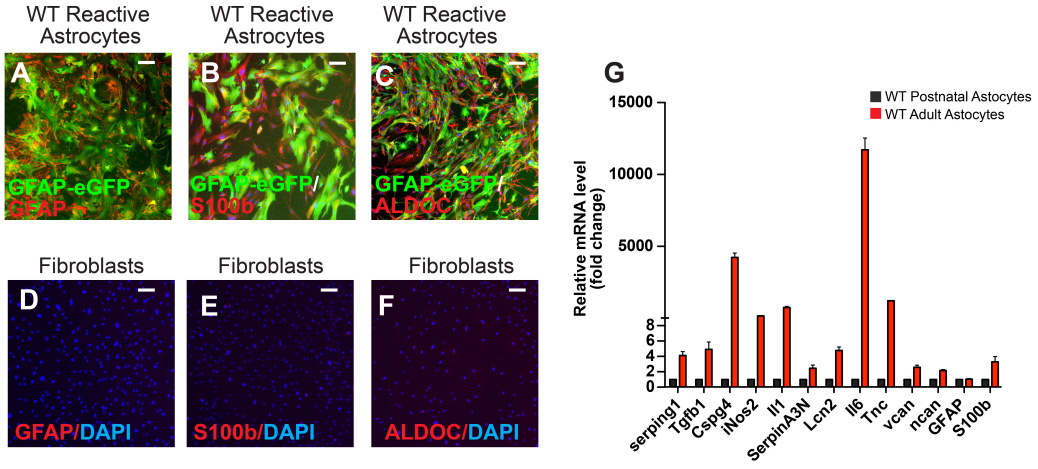

HB9-GFP-WT Motor Neurons
Mutant postnatal Astrocytes
Mutant Adult Astrocytes
P
Q
TAU
TAU

Figure S2: FACS purified human embryonic stem cells derived wild-type hMNs co-cultured with SOD1 mutant adult astrocytes display protein inclusions and axonal swelling (associated with Figure 1 and Figure 2).

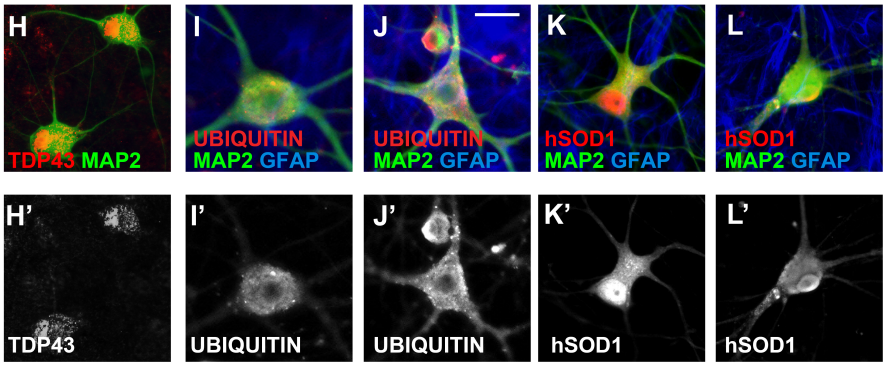
(**A**) Diagram of the motor neuron differentiation protocol. For details, see "Methods" section. **(B)** hES cell colony carrying the HB9-GFP transgene was expanded and differentiated for 14 days showing numerous GFP^+ motor neurons. (**C**) FACS plots of differentiated cell cultures from an HB9-GFP^- hES line. Approximately 20% of the cells were GFP^+.

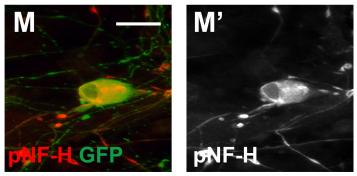
There was no significant difference in the survival and neurite branching of wild-type hMNs co-cultured with either mutant postnatal astrocytes (**D**) or mutant adult astrocytes (**E**) at day 3, suggesting that the initial attachment, survival, and branching of hMNs are comparable in either condition. Quantifications are presented as mean±s.e.m from three separate experiments (**F, G**).

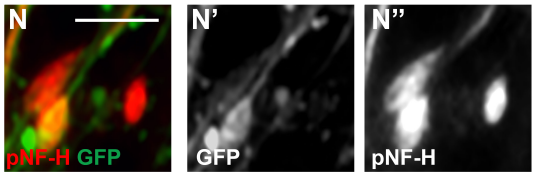
Additional examples of $UBIQUITIN^+$, $P62^+$, pNF-H^+ protein inclusion formation in wild-type human motor neurons co-cultured with mutant adult astrocytes for 60 days (**H-O'**). Abnormal axonal swellings were revealed by TAU staining in wild-type human motor neurons co-cultured with SOD mutant adult astrocytes (**Q**, arrowheads) in contrast to co-cultures with mutant postnatal astrocytes (**P**).



HB9-GFP-WT motor neurons cocultured with wild-type reactive astrocytes







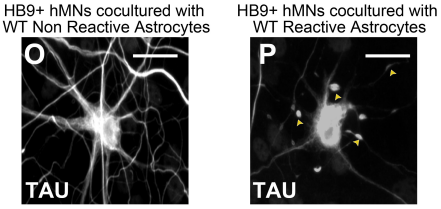


Figure S3. Wild-type hMNs co-cultured with reactive astrocytes isolated from the cortex of stab injury models of adult mice display protein inclusions and axonal swelling (associated with Figure 3).

(**A-F**) Immunohistochemistry detected robust expression of GFAP, S100b, and ALDOC of wild-type reactive astrocytes isolated from adult mice (**A-C**). No expression of these genes was seen in control fibroblasts (**D-F**). qPCR analysis of cultured wild-type adult reactive astrocyte. Compared with early postnatal astrocytes, the adult reactive astrocytes have significantly elevated expression of multiple "reactive" factors (**G**).

(**H-N**) Additional examples of $TDP43^{+}$, $UBIQUITIN^{+}$, $hSOD1^{+}$, and p-NF-H inclusions in wild-type human motor neurons co-cultured with reactive astrocytes for 60 days. (**P**) Abnormal axonal swellings observed by TAU staining in wild-type human motor neurons co-cultured with WT reactive astrocytes (**O**, arrowheads) in comparison to non-reactive astrocytes.

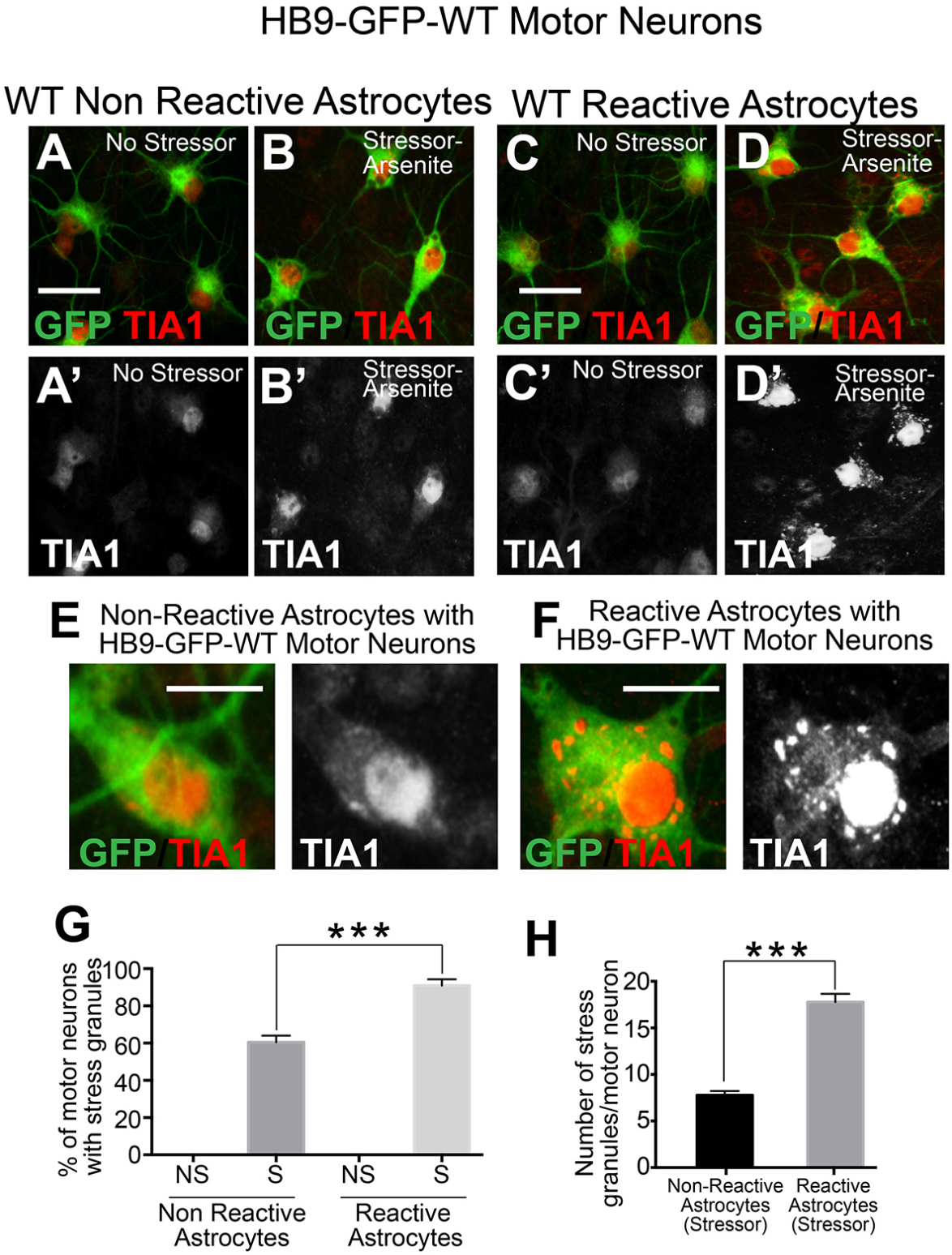

HB9-GFP-WT Motor Neurons
WT Non Reactive Astrocytes
WT Reactive Astrocytes
A
No Stressor
GFP TIA1
B
Stressor-Arsenite
GFP TIA1
C
No Stressor
GFP TIA1
D
Stressor-Arsenite
GFP TIA1
A'
No Stressor
TIA1
B'
Stressor-Arsenite
TIA1
C'
No Stressor
TIA1
D'
Stressor-Arsenite
TIA1
E
Non-Reactive Astrocytes with HB9-GFP-WT Motor Neurons
GFP TIA1
TIA1
F
Reactive Astrocytes with HB9-GFP-WT Motor Neurons
GFP TIA1
TIA1
G
% of motor neurons with stress granules
100
80
60
40
20
0
***
NS
S
NS
S
Non Reactive Astrocytes
Reactive Astrocytes
H
Number of stress granules/motor neuron
20
15
10
5
0
***
Non-Reactive Astrocytes (Stressor)
Reactive Astrocytes (Stressor)

Figure S4. WT reactive astrocytes enhance stress granule formation in human motor neurons (associated with Figure 3).

In 3-day co-cultures of hMNs and astrocytes, the acute stress inducer Arsenite was used to induce stress granules (SG). Immunostaining with the SG marker TIA1 showed that in co-cultures with reactive astrocytes, there was a significant increase in the number of hMNs bearing SGs (**C, D, F, G**), as well as increased number of SGs per motor neuron (**C, D, F, H**), compared with controls (**A, B, E, G, H**). These data suggest increased environmental stress on hMNs from the reactive astrocytes. Quantifications are presented as mean±s.e.m. Data collected from at least three independent experiments each with triplicates. Three asterisks ($P<0.001$). Mann-Whitney test.

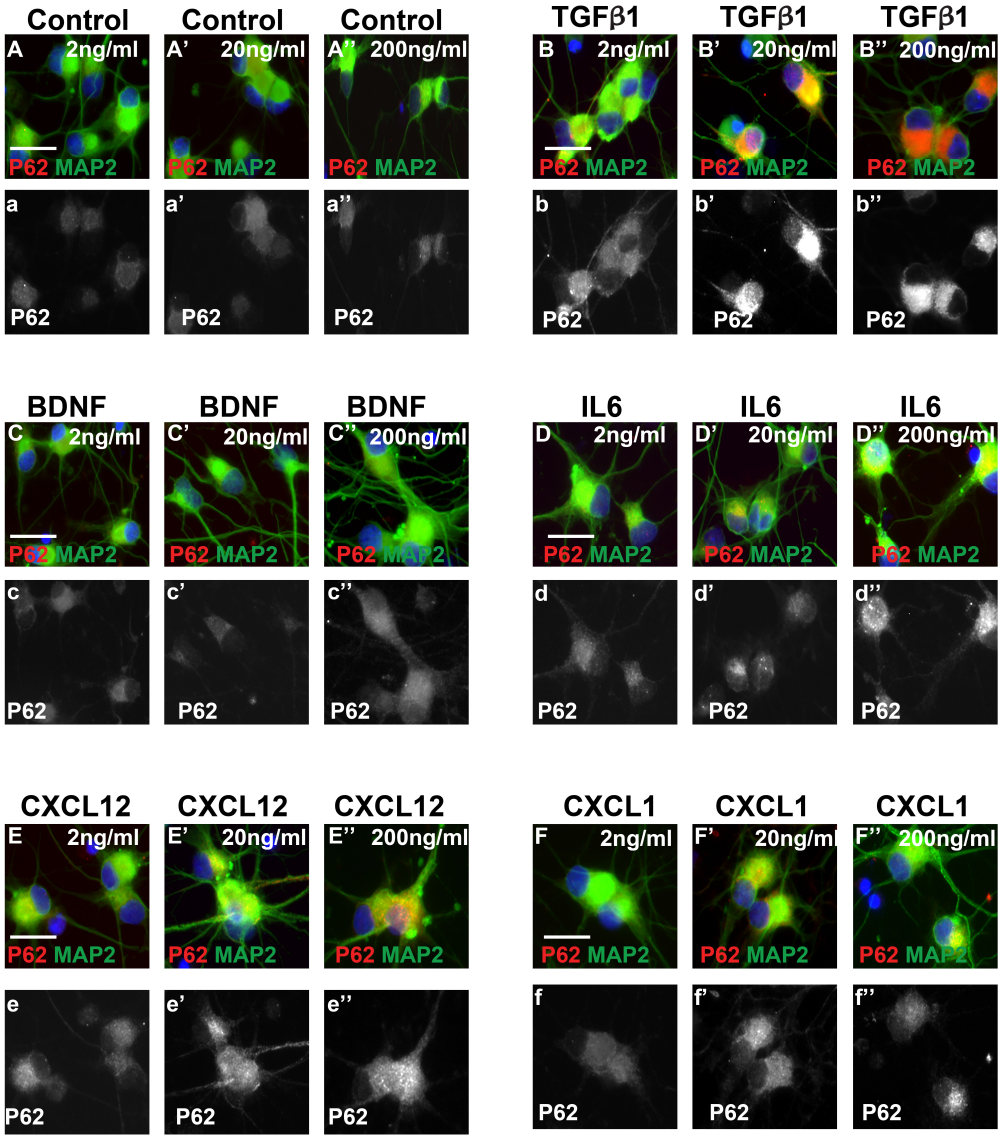


Figure S5. Dose response of P62 inclusion formation in wild-type human motor neurons treated with various cytokines for 14 days (associated with Figure 4).

At 2ng/ml, none of the cytokine-treated hMNs showed significant P62 inclusions. At 20ng/ml, TGFβ1 induced large numbers of P62 inclusions in hMNs whereas IL6, CXCL1, and CXCL12 induced a small number of P62 aggregates. Inclusion formation did not appear to increase significantly at 200ng/ml for CXCL1 and CXCL12, but more were seen in IL6-treated hMNs (albeit less than that of TGFβ1-treated samples).

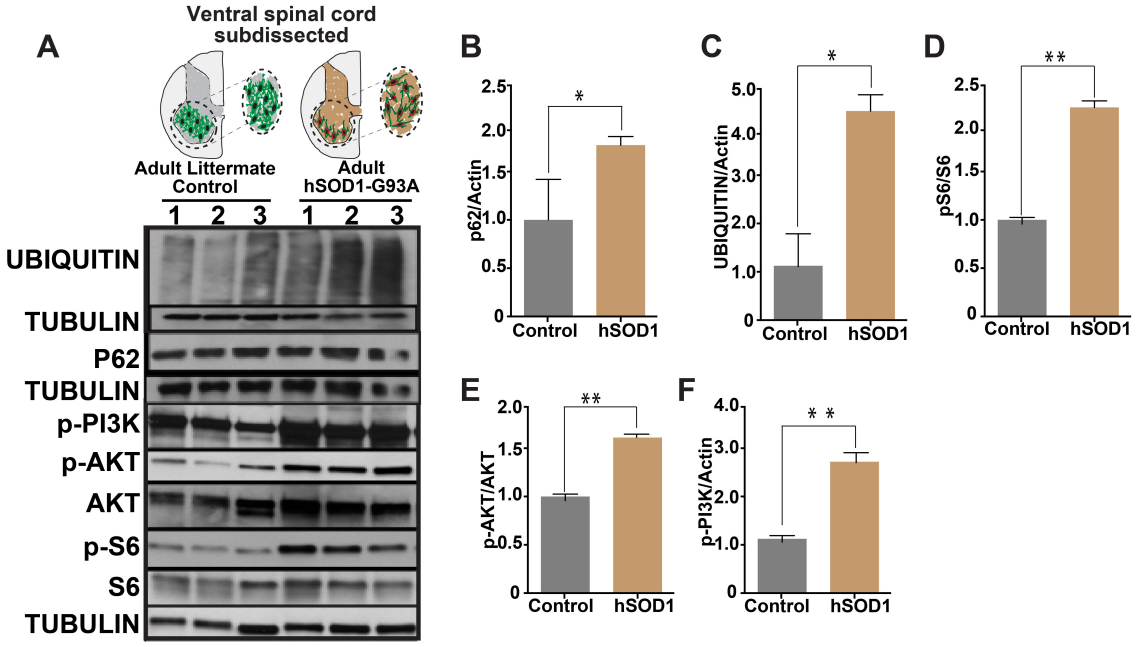


Figure S6. P62 mRNA levels in wild-type human motor neurons treated with various cytokines for 14 days (associated with Figure 6).

The ventral spinal cord tissues of wild-type control mice and hSOD1^{G93A} mutant mice were micro-dissected and analyzed with Western Blot (**A**). P62 and UBIQUITIN levels were significantly increased in the mutant animals (**A-C**). Significant increase of p-S6, p-AKT, and p-PI3K were detected in late stage hSOD1^{G93A} mutant mice, indicating activation of mTOR-PI3K-AKT signaling (**D-F**). Data collected from three independent experiments each with triplicates. One asterisk ($P<0.05$); two asterisks ($P<0.01$). Mann-Whitney test.

Supplemental Tables

Table S1: RT primer sequences (Related to Figure 1 and Figure S3)

Name of the RT primers	Sequence
Gfap iso1_Fw	*acagactttctccaacctccag*
Gfap iso1_Rw	*ccttctgacacggatttggt*
Nf1a-isoA_Fw	*ccagaacttggtggatgga*
Nf1a-isoA_Rw	*gaaccatgtgtaggcgaagg*
S100b_Fw	*aacaacgagctctctcacttcc*
S100b_Rw	*ctccatcactttgtccacca*
Aldoc_Fw	*cgtaggcatcaaggttgaca*
Aldoc_Rw	*gagcacagcgttccaagag*
Aldh1l1_Fw	*tccctacttcccgtctttga*
Aldh1l1_Rw	*acaggctctgcccgattac*
Glt1_iso1&3_Fw	*ttctacagctgagagaatggtca*
Glt1_iso1&3_Rw	*ttcggtgctttggctcat*
Aqp4_Fw	*tggaggattgggagtcacc*
Aqp4_Rw	*tgaacaccaactggaaagtga*
Glast_Fw	*agaaggtaaaatcgtgcaggtc*
Glast_Rw	*accagattgggagggaacat*
Glul_Fw	*ctcgctctcctgacctgttc*
Glul_Rw	*ttcaagtgggaacttgctga*
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Vim_Rw	*tgcgccagcagtatgaaa*
Acan_Fw	*gagggtgggaagccatgt*
Acan_Rw	*ccagcctacaccccagtg*
Vcan_Fw_iso1	*cagcggcaaagttcagagt*
Vcan_Rw_iso1	*cactggctgtggatggtg*
Ncan_Fw	*gcttcgacgcctactgctt*
Ncan_Rw	*tccagatgaggggatctcag*
Cspg4_Fw	*cacctccaggtggttctcc*
Cspg4_Rw	*cttggccttgttggtcagat*
Lcn2_Fw	*tctgatccagtagcgacagc*
Lcn2_Rw	*ccatctatgagctacaagagaacaat*
Serpina3n_Fw	*acatcgggagtcagctatcac*
Serpina3n_Rw	*ccatcttctgtgttctgcagtc*
IL6_Fw	*ccaggtagctatggtactccagaa*
IL6_Rw	*gctaccaaactggatataatcagga*
Tgfb1_Fw	*gtcagcagccggttacca*
Tgfb1_Rw	*tggagcaacatgtggaactc*
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Serping1_Fw	*ccaaaggtgtcacttctgtgtc*
Serping1_Rw	*gagatgcattcacataggtgtcc*
Cxcl12_Rw	*ctgtgcccttcagattgttg*
Cxcl12_Fw	*ctctgcgccccttgttta*
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Stat6_Rw	*ctgcgaacccttgtgacc*
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