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Supplemental Information

Hexanucleotide Repeat Expansions in c9FTD/ALS and SCA36 Confer Selective Patterns of Neurodegeneration *In Vivo*

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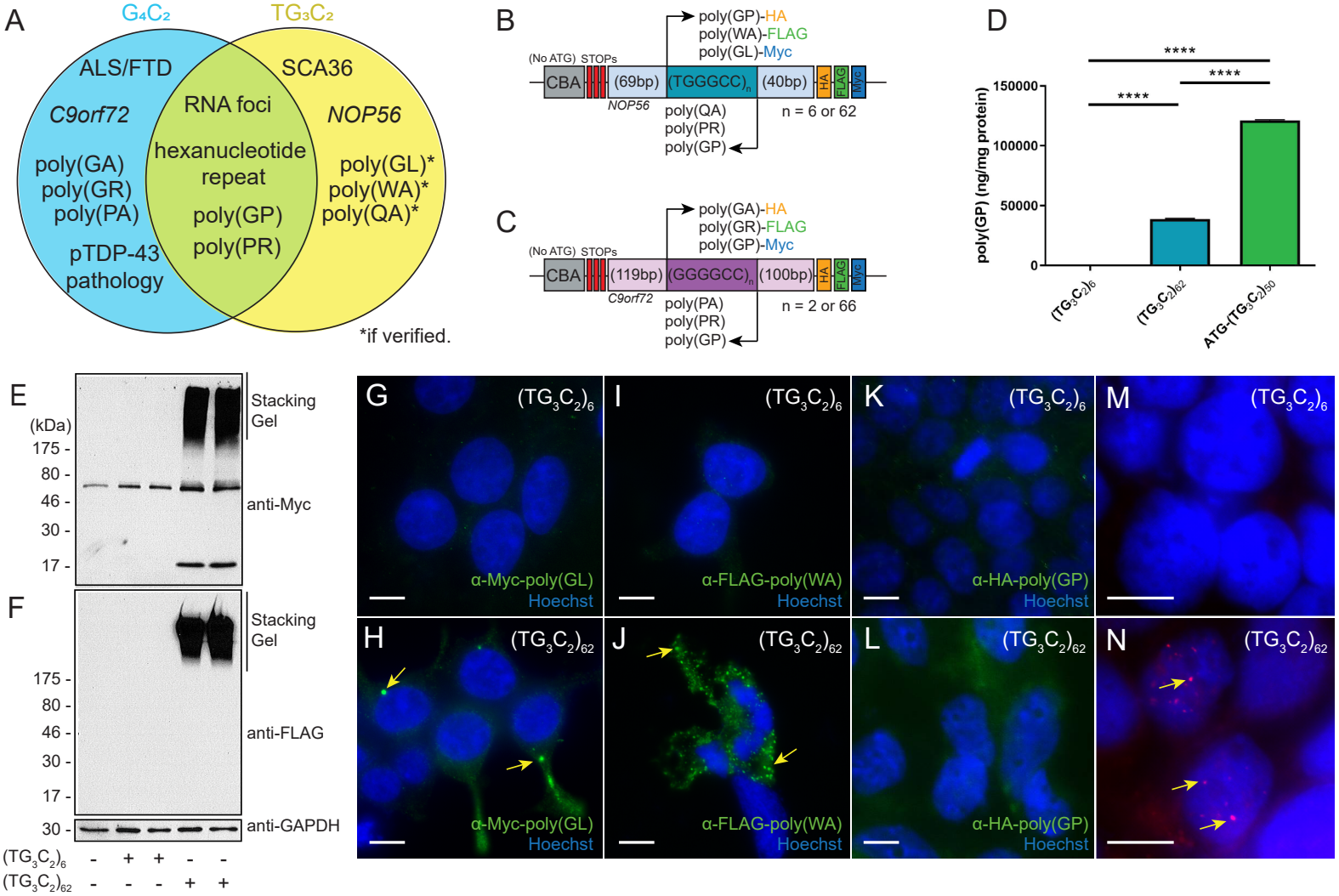
A
G4C2
TG3C2
ALS/FTD
C9orf72
poly(GA)
poly(GR)
poly(PA)
pTDP-43
pathology
RNA foci
hexanucleotide
repeat
poly(GP)
poly(PR)
SCA36
NOP56
poly(GL)*
poly(WA)*
poly(QA)*
*if verified.
B
poly(GP)-HA
poly(WA)-FLAG
poly(GL)-Myc
(No ATG) STOPs
CBA
(69bp)
(TGGGCC)n
(40bp)
HA
FLAG
Myc
NOP56
poly(QA)
poly(PR)
poly(GP)
n = 6 or 62
C
poly(GA)-HA
poly(GR)-FLAG
poly(GP)-Myc
(No ATG) STOPs
CBA
(119bp)
(GGGGCC)n
(100bp)
HA
FLAG
Myc
C9orf72
poly(PA)
poly(PR)
poly(GP)
n = 2 or 66
D
poly(GP) (ng/mg protein)
150000
100000
50000
0
****
****
****
(TG3C2)6
(TG3C2)62
ATG-(TG3C2)50
E
(kDa)
175 -
80 -
46 -
30 -
17 -
Stacking
Gel
anti-Myc
F
175 -
80 -
46 -
30 -
17 -
30 -
Stacking
Gel
anti-FLAG
anti-GAPDH
(TG3C2)6 - + + - -
(TG3C2)62 - - - + +
G
(TG3C2)6
α-Myc-poly(GL)
Hoechst
I
(TG3C2)6
α-FLAG-poly(WA)
Hoechst
K
(TG3C2)6
α-HA-poly(GP)
Hoechst
M
(TG3C2)6
H
(TG3C2)62
α-Myc-poly(GL)
Hoechst
J
(TG3C2)62
α-FLAG-poly(WA)
Hoechst
L
(TG3C2)62
α-HA-poly(GP)
Hoechst
N
(TG3C2)62

Figure S1: Expanded TG_3C_2 repeats undergo RAN translation *in vitro* in HEK293T cells. Related to Figures 1-4.

(A) c9FTD/ALS and SCA36 are distinct neurodegenerative disorders, but they are caused by highly similar hexanucleotide repeat expansions. This Venn diagram compares and contrasts these two repeat expansions. Both the G_4C_2 and TG_3C_2 repeats can form RNA foci and both encode the DPRs poly(GP) and poly(PR), but each repeat also encodes divergent DPRs, and only the G_4C_2 repeat has been associated with the development of pTDP-43 pathology.

(B and C) Schematic diagrams of the TG_3C_2 (B) and G_4C_2 (C) repeat constructs used in this study. Both contain flanking sequences from the endogenous human gene, as well as protein tags that are in-frame with the different sense DPRs. Neither construct contains an ATG start site.

(D) An MSD immunoassay was used to measure poly(GP) levels in lysates from HEK293T cells transfected with the indicated repeat constructs. Values represent the average amount of poly(GP) detected over replicates, minus background levels from untransfected and EGFP-transfected control cells. Error bars are SEM over 3 replicate experiments. ****$p \leq 0.0001$ (1-way ANOVA with Tukey's multiple comparisons test.)

(E and F) Western blot analysis of whole cell lysates from HEK293T cells transiently transfected with the indicated repeat constructs. Lysates were assessed for both the Myc tag (E) and the FLAG tag (F). GAPDH was used as a loading control. kDa = kiloDalton.

(G-L) IF for the protein tags in HEK293T cells expressing the control (G,I,K) or expanded (H,J,L) TG_3C_2 repeat. The tagged DPR detected in each panel is marked in green; nuclei were labeled with Hoescht in blue. Arrows mark inclusions. For anti-Myc, we generally observe 1-2 puncta per cell. For anti-FLAG, there are often multiple puncta per cell as shown, but some cells show only one large perinuclear aggregate.

(M and N) FISH was used to detect sense RNA foci (arrows) in HEK293T cells that were transiently transfected with $(TG_3C_2)_6$ (M) or $(TG_3C_2)_{62}$ (N). DAPI marks nuclei in blue. All scale bars are 10 μm.

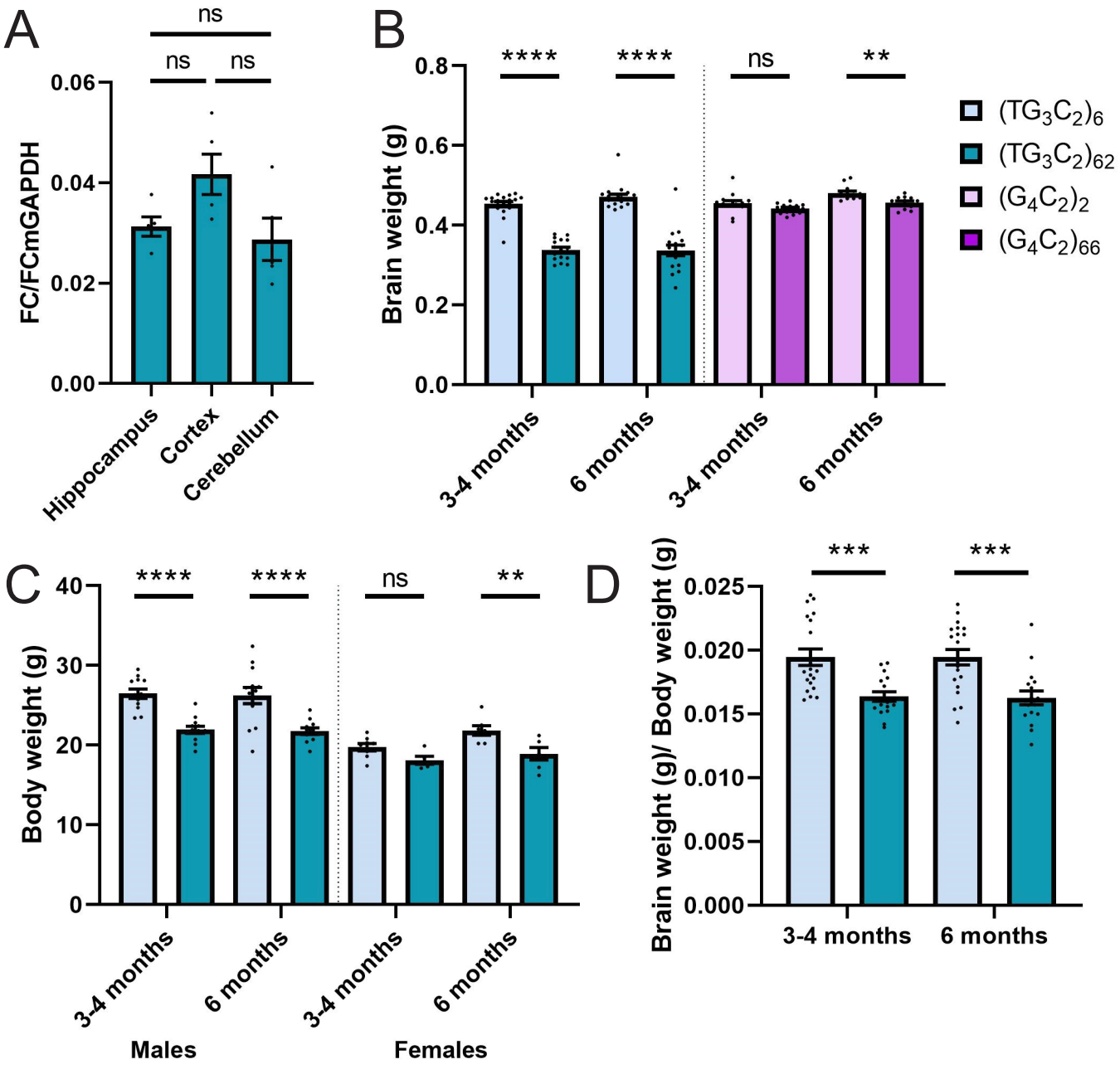
A
ns
ns
ns
FC/FCmGAPDH
0.06
0.04
0.02
0.00
Hippocampus
Cortex
Cerebellum
B
****
****
ns
**
Brain weight (g)
0.8
0.6
0.4
0.2
0.0
3-4 months
6 months
3-4 months
6 months
(TG3C2)6
(TG3C2)62
(G4C2)2
(G4C2)66
C
****
****
ns
**
Body weight (g)
40
30
20
10
0
3-4 months
6 months
3-4 months
6 months
Males
Females
D
***
***
Brain weight (g)/ Body weight (g)
0.025
0.020
0.015
0.010
0.005
0.000
3-4 months
6 months

Figure S2: $(TG_3C_2)_{62}$ mice show decreased brain and body weight compared to controls. Related to Figure 1.

(A) qRT-PCR was used to detect $(TG_3C_2)_{62}$ transcript levels in different regions of the mouse brain as indicated. Data shown uses primers directed against the WPRE domain of the AAV transgene, but similar results were obtained using primers against the *NOP56* flanking sequences. Primers against mGAPDH were used as an endogenous control. *n* = 5-6 mice per brain region. ns = non-significant (1-way ANOVA with Tukey's multiple comparisons test).

(B) Brain weight at harvest for the different mouse models indicated. For 3- to 4-month-old mice: *n* = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$; 17 $(G_4C_2)_2$; and 12 $(G_4C_2)_{66}$. For 6-month-old mice: *n* = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$; 14 $(G_4C_2)_2$; and 12 $(G_4C_2)_{66}$.

(C) Average body weight in grams of the TG_3C_2 repeat mice at each time point. Data is divided by sex as male mice are naturally larger than females. For 3- to 4-month-old mice: *n* = 12M, 8F $(TG_3C_2)_6$; 12M, 5F $(TG_3C_2)_{62}$. For 6-month-old mice: *n* = 13M, 7F $(TG_3C_2)_6$; 11M, 6F $(TG_3C_2)_{62}$.

(D) Brain weight divided by body weight for the TG_3C_2 mouse model. For 3- to 4-month-old mice: *n* = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$. For 6-month-old mice: *n* = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$.

For (B-D), error bars are SEM. **p ≤ 0.01, ***p ≤ 0.001, ****p ≤ 0.0001, ns = non-significant (2-way ANOVA with Sidak's multiple comparisons test). All bar graphs use the same color scheme, and legends are therefore only shown once per figure.

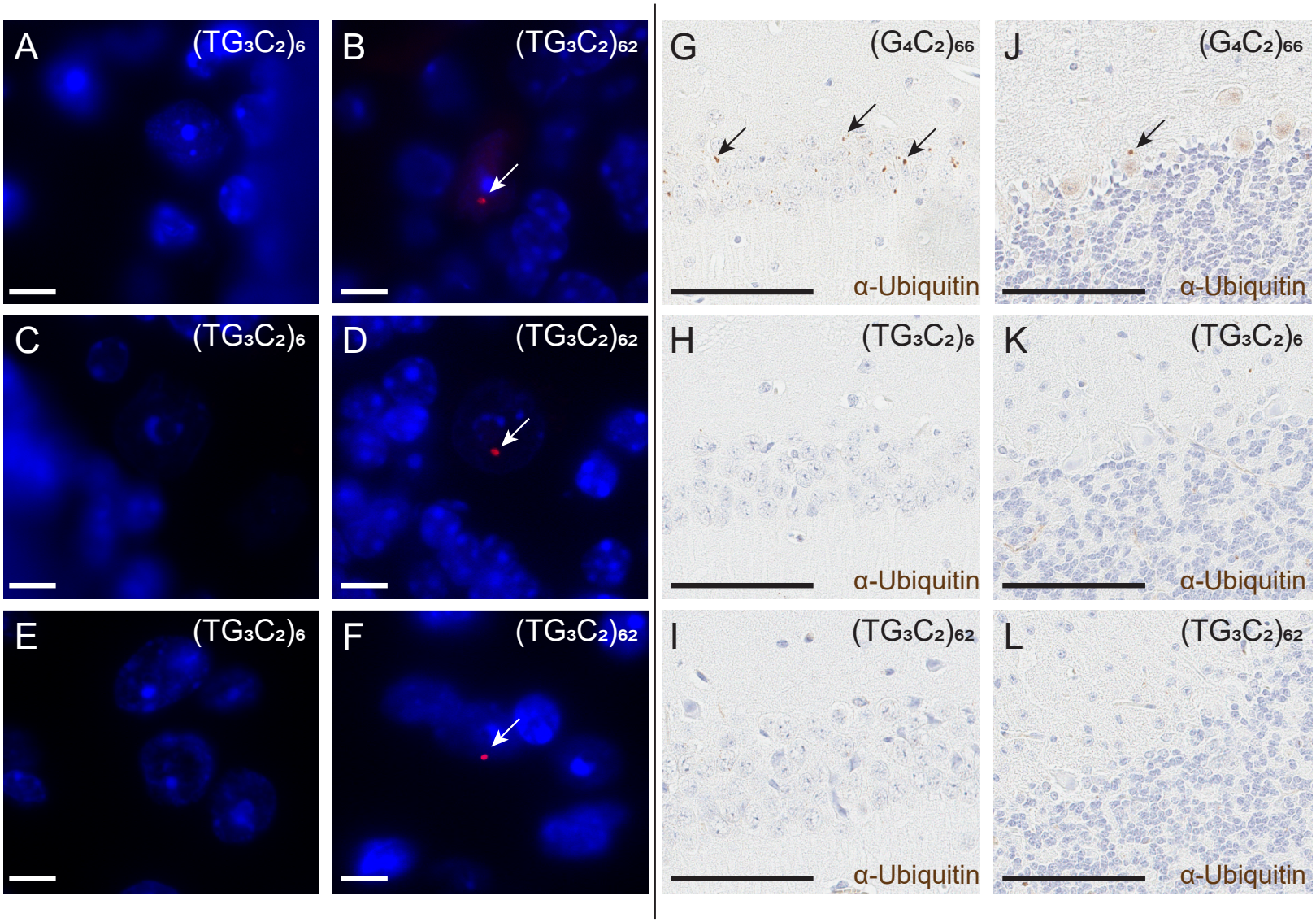
A
(TG3C2)6
B
(TG3C2)62
C
(TG3C2)6
D
(TG3C2)62
E
(TG3C2)6
F
(TG3C2)62
G
(G4C2)66
α-Ubiquitin
J
(G4C2)66
α-Ubiquitin
H
(TG3C2)6
α-Ubiquitin
K
(TG3C2)6
α-Ubiquitin
I
(TG3C2)62
α-Ubiquitin
L
(TG3C2)62
α-Ubiquitin

Figure S3: Rare antisense RNA foci were detected in the $(TG_3C_2)_{62}$ mice, but no ubiquitin-positive inclusions were observed. Related to Figure 3.

(A-F) FISH was used to detect antisense RNA foci in the brains of $(TG_3C_2)_6$ (A,C,E) and $(TG_3C_2)_{62}$ (B,D,F) mice. Antisense foci were very rare, but most often observed in the cerebellar Purkinje cells. Representative images from the cerebellum of 6-month-old (A,B) and 12-month-old (C,D) mice are shown, as well as an example from the cortex of a 12-month-old mouse (E,F). DAPI marks nuclei in blue. Scale bars are 10 μm.

(G-L) IHC for ubiquitin in the brains of 3- to 4-month-old mice expressing $(G_4C_2)_{66}$ (G,J), the $(TG_3C_2)_6$ control (H,K), or $(TG_3C_2)_{62}$ (I,L). Representative images from the hippocampus (G-I) and cerebellum (J-L) are shown. Similar results were observed in 6-month-old animals. Arrows mark inclusions. Scale bars are 100 μm.

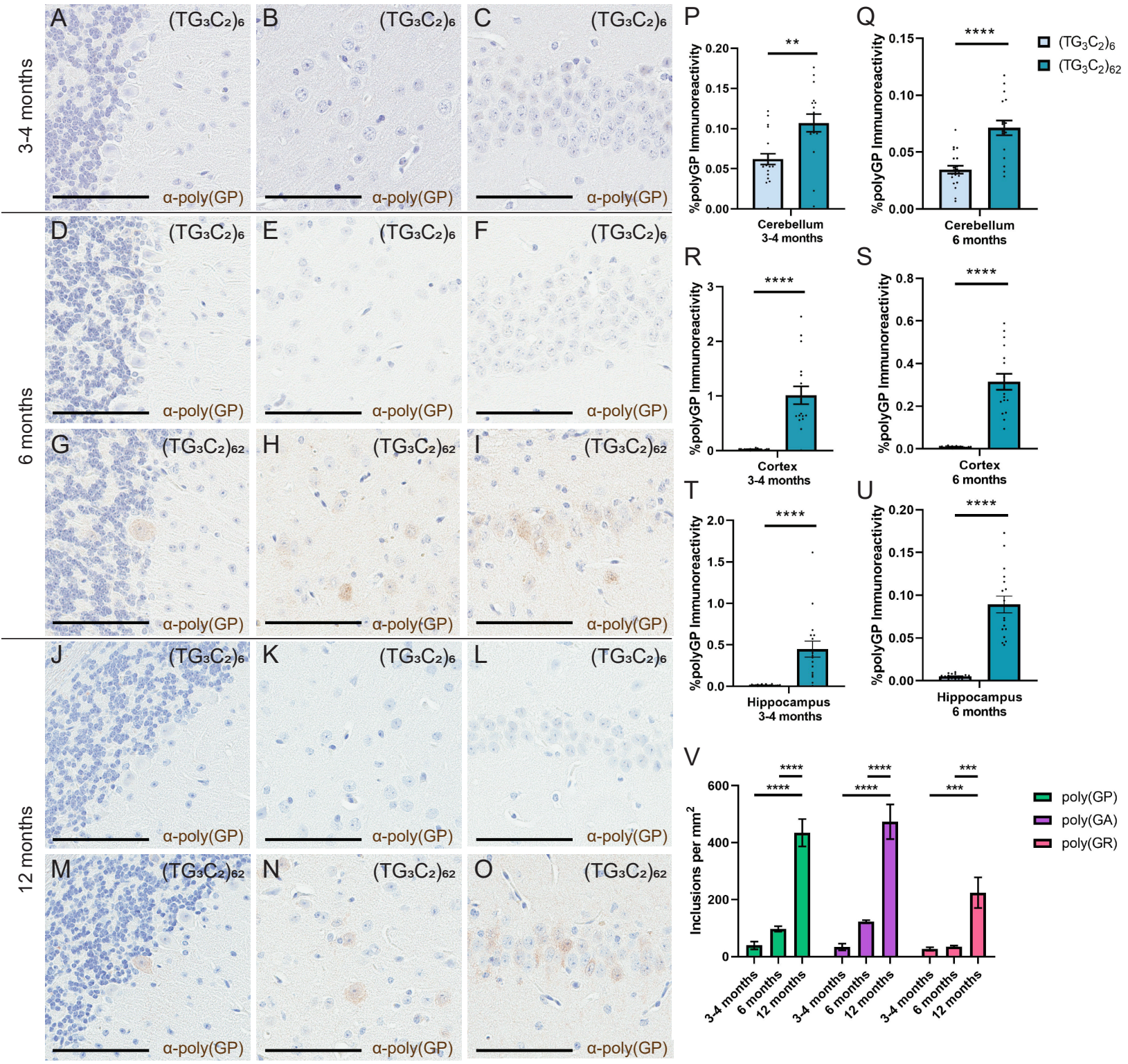
3-4 months
6 months
12 months
A
B
C
D
E
F
G
H
I
J
K
L
M
N
O
(TG3C2)6
(TG3C2)62
α-poly(GP)
P
Q
R
S
T
U
V
%polyGP Immunoreactivity
Cerebellum 3-4 months
Cerebellum 6 months
Cortex 3-4 months
Cortex 6 months
Hippocampus 3-4 months
Hippocampus 6 months
Inclusions per mm2
poly(GP)
poly(GA)
poly(GR)
3-4 months
6 months
12 months

Figure S4: Poly(GP) displays a diffuse localization pattern in $(TG_3C_2)_{62}$ mice at all ages analyzed, but forms age-dependent inclusions in $(G_4C_2)_{66}$ mice. Related to Figure 3.

(A-C) Representative images from the cerebellum (A), cortex (B) and hippocampus (C) of $(TG_3C_2)_6$ mice immunostained for poly(GP) at 3-4 months of age.

(D-I) Representative images from the cerebellum (D,G), cortex (E,H) and hippocampus (F,I) of $(TG_3C_2)_6$ (D,E,F) and $(TG_3C_2)_{62}$ (G,H,I) mice immunostained for poly(GP) at 6 months of age.

(J-O) Representative images from the cerebellum (J,M), cortex (K,N) and hippocampus (L,M) of $(TG_3C_2)_6$ (J,K,L) and $(TG_3C_2)_{62}$ (M,N,O) mice immunostained for poly(GP) at 12 months of age. Scale bars are 100 μm.

(P-U) The degree of diffuse poly(GP) immunoreactivity in the cerebellum (P,Q), cortex (R,S), and hippocampus (T,U) of the TG_3C_2 repeat mice was quantified at both 3-4 months (P,R,T) and 6 months (Q,S,U) of age. Error bars are SEM. For 3- to 4-month-old mice: n = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$. For 6-month-old mice: n = 20 $(TG_3C_2)_6$; 17 $(TG_3C_2)_{62}$. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$ (unpaired two-tailed student's t-test).

(V) Brain sections from $(G_4C_2)_{66}$ mice were immunostained for poly(GP), poly(GA) or poly(GR). The number of inclusions per millimeter in the motor cortex of the mice was counted at each time point listed. Error bars are SEM. n = 8 per time point. $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$ (2-way ANOVA with Tukey's post-hoc multiple comparisons test.)

Gender	Age of onset	Age at autopsy	Survival in years	Clinical Diagnosis	Genetic Diagnosis	Country of Origin
M	60	91	31	SCA	SCA36	Spain
M	60	95	35	SCA	SCA36	Spain
F	63	77	14	SCA	SCA36	Spain
M	58	71	13	ALS/FTLD-TDP	c9FTD/ALS	USA

SCA = spinocerebellar ataxia; SCA36 = spinocerebellar ataxia type 36

ALS = amyotrophic lateral sclerosis

FTLD-TDP = frontotemporal lobar degeneration with TDP-43 inclusions

c9FTD/ALS = *C9orf72*-associated frontotemporal dementia and ALS

Table S1: Patient information on post-mortem samples analyzed. Related to Figure 3.

Similar results were seen in all SCA36 patient samples. The representative image included in Figure 3P is from the first patient listed. The representative image in Figure 3R is from the second. Poly(GP) and poly(PR) pathology observed in the brain of this c9FTD/ALS patient is consistent with other patient samples analyzed in the literature.