FUS-regulated region- and cell-type-specific transcriptome is associated with cell selectivity in ALS/FTLD

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SUPPLEMENTARY INFORMATION

Supplementary results

Differentially expressed genes in *Fus*-silenced cells of the CNS

The gene expression profiles of Fus-silenced cells of the CNS were analyzed. First, we filtered genes by signal intensity with the t-test (p value ≤ 0.1). Table S3 lists the top 10 genes that were differentially expressed in Fus-silenced primary motor neurons, together with the fold-change values in primary cortical neurons, glial cells, and cerebellar neurons. Similar to the global profile comparison shown in Figure 2, the expression patterns of genes in the three different primary cells were similar except for primary cerebellar neurons, whose profile was less altered by Fus-depletion than other primary cell types.

Comparison of FUS-binding frequency in genes with altered alternative splicing events and gene expression

To compare the frequency of FUS-binding with genes of altered alternative splicing events and gene expression, we analyzed the frequency of CLIP-tags in 2,175 genes with altered FUS-responsive exons and 2,347 genes with altered gene expression after Fus-silencing with shFus1 (filtered by t-test p value of ≤ 0.1). Plot analysis of FUS tags showed significantly larger number of tags per 1 kbp pre-mRNA in alternative splicing than in gene expression

(Figure S4, left). Genes without FUS tags were more common in gene expression than in alternative splicing (462 for gene expression, 67 for alternative splicing). Density comparison analysis also indicated that genes with altered alternative splicing events had more frequenct FUS-tags than genes with altered gene expression (Figure S4, right).

Supplementary experimental procedures

Primary cells from the central nervous system

Primary cortical neurons were prepared as described previously [1]. Primary motor neurons were harvested from mouse spinal cords of C57BL/6 strain mouse embryos at embryonic (E) day 13. Dorsal root ganglia and the dorsal half of the spinal cord were removed as well as meninges. The ventral portion of the spinal cord was cut in approximately 1-mm pieces and dissociated into a single-cell suspension using Sumilon dissociation solution (Sumitomo Bakelite, Tokyo).

Motor neurons were harvested by gradient centrifugation with Optiprep (Invitrogen). Cells were plated at a density of 1.5 x 10^6 cells in a 60 mm-well culture plate with a medium containing 0.5x Sumilon nerve-culture medium (Sumitomo Bakelite), 0.5 x Neurobasal medium, 1% fetal bovine serum (FBS), 0.5 x B27 supplements (Invitrogen), 0.5 x Glutamax, 5 μg/ml of brain-derived neurotrophic factor (BDNF), 5 μg/ml of ciliary neurotrophic factor (CNTF), and 0.5% Pen-Strep. One day after plating (day 2), neurons were supplemented with 10 ng/μl of AraC and incubated overnight. On day 5, neurons were infected with 2 x 10^{10} copies/well (1.5 x 10^7 copies/μl) of lentivirus expressing shRNA against mouse *Fus* (shFUS1 or shFUS2) or control (shCont). After 4 hr of infection, the virus medium was removed. Neurons were then cultured for 6 additional days, and were harvested on day 11 followed by RNA extraction and cDNA synthesis.

For primary glial cells, mouse brains were harvested from C57BL/6 strain mouse embryos at E15. The meninges-free cerebral cortices were extracted and dissociated into a single-cell suspension using Sumilon dissociation solution (Sumitomo Bakelite, Tokyo). Cells were plated at a density of 1.5 x 10^6 cells in a 60 mm-well culture plate with Dulbecco's Modified Eagle Medium (D-MEM) containing 10% FBS and 1% Pen-Strep. On day 11, glial cells were infected with 1.81 x 10^{10} copies/well (1.36 x 10^7 copies/μl) of lentivirus expressing shRNA against mouse *Fus* (shFUS1 or shFUS2) or control (shCont). After overnight infection, the virus medium was removed. Glia cells were cultured for 5 days after the infection and RNA was extracted on day 16.

Primary cerebellar neurons were obtained from the cerebellum of C57BL/6 strain mouse embryos at E15. The meninges-free cerebellum were extracted and dissociated by Sumilon dissociation solution (Sumitomo Bakelite). Cells were plated at a density of 1.7 x 10^6 cells in a 60 mm-well culture plate with Sumilon nerve-culture medium (Sumitomo Bakelite). On day 5, cerebellar neurons were infected with 2.5 x 10^{10} copies/well (1.25 x 10^7 copies/μl) of lentivirus expressing shRNA against mouse *Fus* (shFUS1 or shFUS2) or control (shCont). After 4 hr of infection, the medium containing the virus was removed. Neurons were then cultured for 4 additional days, and harvested on day 9 followed by RNA extraction and cDNA synthesis.

Each knock-down experiment was performed in triplicate for each microarray analysis. For immunoblot, we used anti-FUS antibody (A300-293A, Bethyl Laboratories), and anti-actin antibody (Sigma) were used. For immunohistochemistry, we used anti-FUS antibody (A300-293A, Bethyl Laboratories), anti-β tubulin antibody (TU20, Santa Cruz Biotechnology), anti-neurofilament H non-phosphorylated (SMI 32, Covance, Tokyo) antibody, anti-GFAP antibody (EB4, Enzo Life Sciences, Plymouth Meeting, PA), anti-Calbindin antibody (Abcam), anti-Zic1 antibody (Abcam), and DAPI.

Real-time qPCR for gene expression analysis

Total RNA was isolated from cells using RNeasy Mini Kit (Qiagen) and reverse transcribed using 1 μg of total RNA with Oligo-dT primer. For the thermal cycle reaction, the CFX96 system (BioRad) was used at 95°C for 3 min, then 40 cycles at 95°C for 10 sec and 55°C for 30 sec. The relative amount for each transcript was calculated by drawing a standard curve of cycle thresholds for serial dilutions of cDNA samples and normalized to the amount of β-actin. The PCR was performed in triplicate for each sample, after which all experiments were repeated twice. The sets of primers (Table S4) and iQ SYBR Green Supermix (BioRad) were used for real-time qPCR.

Bioinformatics analysis

We reanalyzed HITS-CLIP data that were reported previously by our group from cerebrum obtained from 12-week-old C57BL/6 mice [1]. The HITS-CLIP data are available in the NCBI Gene Expression Omnibus database (accession number GSE37190). All CLIP-tags on the whole transcript or on three exons spanning an alternatively spliced exon were summed to calculate frequency of CLIP-tags per kb of pre-mRNA. Statistical analysis was performed using JMP software version 9.0.2 (SAS Institute Inc, Cary, NC). Normalized complexity maps of FUS-RNA interactions were generated as described previously[2]. For the control, normalized complexity map was similarly generated by analyzing 100 sets of 20 constitutive exons that were randomly selected from 118,969 constitutive exons in the mouse genome.

To identify enriched Gene Ontology terms, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID 6.7)[3,4].

Supplementary Figure legends

Figure. S1 Efficient transduction of shRNA against *Fus* into different cell lineages from the central nervous system using lentivirus. (A) *Fus* was efficiently knocked down in all four primary cells from the central nervous system, as evaluated by real-time qRT-PCR. Bars: mean±SD. Experiments were performed in triplicate. **(B)** Immunoblot analysis showed that the protein levels of FUS were decreased after transduction of shRNA in all four primary cells from the central nervous system. **(C)** Immunohistochemical analysis using anti-FUS antibody and primary motor neurons (top left), primary cortical neurons (top right), primary glial cells (bottom left), and primary cerebellar neurons (bottom right) silenced by shFUS1, shFUS2, and shCont. Cells were fixed and immunostained with anti-FUS antibody, DAPI, and marker antibodies: anti-Neurofilament-H antibody (SMI32R) for primary motor neurons, anti-β tubulin antibody (TU20) for primary cortical neurons, anti-GFAP antibody for glial cells, and anti-Zic1 antibody for granule cells in primary cerebellar neurons (bottom right).

Figure. S2 Scatter plots of fold changes in gene expression levels and alternative splicing events in primary motor neurons, cortical neurons, and glial cells after *Fus* knock-down. Fig 2 showed the fold-changes in overlapped genes and exons filtered by the t-test ($p<0.1$) among four different primary cells. With the t-test of lower *p* value ($p<0.075$ and 0.05), the fold-changes in overlapped genes and exons were again plotted for motor neurons, cortical neurons, and glial cells. We excluded cerebellar neurons in this analysis, since the number of overlapped genes and exons in cerebellar neurons were too small after filtration by the t-test of lower *p* values ($p<0.075$ and 0.05). **(A)** Scatter plots of fold changes in gene expression levels in four different primary cells from the central nervous system after *Fus* knock-down.

R^2 was calculated by the Student's *t*-test ($p<0.075$ for top rows and <0.05 for bottom rows). **(B)** Scatter plots of fold-changes in alternative splicing events in four different primary cells from the central nervous system after *Fus* knock-down. R^2 was calculated by the Student's *t*-test ($p<0.075$ for top rows and <0.05 for bottom rows). **(C)** Graphical presentations of R^2 scores with different t-test *p* values. Note that stringent t-test *p* values increased the coefficients of determinant (R^2) in datasets. The R^2 values for the correlation between motor/cortical neurons and glial cells are consistently low in splicing profiles.

Figure. S3 Validation of altered splicing events in different cell lineages from the central nervous system after *Fus-silencing*. (A-E) 44 exons with differential expression after Fus-silencing were validated by semi-quantitative RT-PCR (Table 2) and 9 representative alternative splicing events are shown in Fig. 3. All the validated exons with differential expression after Fus-silencing are shown here. The Fus-regulated alternative splicing events are **(A)** both motor and cortical neuron-specific, **(B)** cortical neuron-specific, **(C)** motor neuron-specific **(D)** glial cell-specific, and **(E)** common among motor neurons, cortical neurons, glial cells and/or cerebellar neurons. The top panels represent schematic splicing changes mediated by FUS. shCont and shFUS resulted in splicing events shown in the top and bottom rows, respectively. The second panels show representative RT-PCR of the indicated exons in primary motor neurons. Similarly, the third, fourth, and fifth panels show representative RT-PCR of the indicated exons in primary cortical neurons, primary glial cells, and primary cerebellar neurons, respectively. The experiments were repeated four times using four independent sets of samples. The results of densitometric quantification of RT-PCR are shown in bar graphs (n=4; mean±SD). *$p<0.05$, between shCont and shFUS (by Student's t-test).

Figure. S4 Comparison of FUS-binding frequency in genes with altered alternative splicing events and gene expression The frequency of CLIP-tags was analyzed in 2,175 genes with altered FUS-responsive exons (filtered by t-test *p* value ≤0.1) and 2,347 genes with altered gene expression (filtered by t-test *p* value ≤0.1) after Fus-silencing with shFUS1. The histograms of FUS tags-distribution showed significantly larger number of tags per 1 kbp pre-mRNA in alternative splicing than in gene expression (Wilcoxson test, $p<0.001$, left panel). A larger number of scoreless tags were observed in gene expression than in alternative splicing (462 for gene expression, 67 for alternative splicing). The mean value of FUS tags was 2602.4 in alternative splicing, and 1944.5 in gene expression. Density comparison analysis also indicated that genes with altered alternative splicing events had more frequent FUS-tags than genes with altered gene expression (right panel).

Table S1. Correlation coefficients of gene expression profiles in primary cells infected with shCont.

cell-type	motor neurons	motor neurons	motor neurons	cortical neurons	cortical neurons	cortical neurons	glial cells	glial cells	glial cells	cerebellar neurons	cerebellar neurons	cerebellar neurons
motor neurons	1	0.9895527	0.9917904	0.91768044	0.92028743	0.93367445	0.8376465	0.83593583	0.82761204	0.90170926	0.9106213	0.91927004
motor neurons	0.9895527	1	0.98831934	0.93171865	0.9343572	0.94594234	0.8220201	0.8170681	0.8150147	0.91167986	0.916354	0.92661697
motor neurons	0.9917904	0.98831934	1	0.9283221	0.92997694	0.94393647	0.80601597	0.8027459	0.79517984	0.9049175	0.91566336	0.92314386
cortical neurons	0.91768044	0.93171865	0.9283221	1	0.99698764	0.9845726	0.7208252	0.7183655	0.7227003	0.9166653	0.9158916	0.92024326
cortical neurons	0.92028743	0.9343572	0.92997694	0.99698764	1	0.9843074	0.7263636	0.725308	0.72941077	0.9193453	0.9172309	0.9218666
cortical neurons	0.93367445	0.94594234	0.94393647	0.9845726	0.9843074	1	0.75221545	0.744647	0.7460938	0.920952	0.92191416	0.92797256
glial cells	0.8376465	0.8220201	0.80601597	0.7208252	0.7263636	0.75221545	1	0.99125874	0.9899257	0.7563115	0.7554118	0.7651825
glial cells	0.83593583	0.8170681	0.8027459	0.7183655	0.725308	0.744647	0.99125874	1	0.9893296	0.75168437	0.7508623	0.75995713
glial cells	0.82761204	0.8150147	0.79517984	0.7227003	0.72941077	0.7460938	0.9899257	0.9893296	1	0.75703853	0.75200313	0.76141226
cerebellar neurons	0.90170926	0.91167986	0.9049175	0.9166653	0.9193453	0.920952	0.7563115	0.75168437	0.75703853	1	0.98169434	0.9860409
cerebellar neurons	0.9106213	0.916354	0.91566336	0.9158916	0.9172309	0.92191416	0.7554118	0.7508623	0.75200313	0.98169434	1	0.9954495
cerebellar neurons	0.91927004	0.92661697	0.92314386	0.92024326	0.9218666	0.92797256	0.7651825	0.75995713	0.76141226	0.9860409	0.9954495	1

Table S2. Numbers of differentially expressed genes in each primary cell type.

	total (t-test, <0.1)	up (FC>1.3)	down (0.77>FC)
motor neurons	2321	251	176
cortical neurons	2470	454	481
glial cells	2074	205	157
cerebellar neurons	494	23	14

Table S3. Top 10 genes down- and up-regulated by FUS-silencing in each primary cell type. Genes were first filtered by signal intensity with the t-test (*p* value ≤0.1). The list shows the top 10 genes that were differentially expressed in Fus-silenced primary motor neurons, together with the fold-change values in primary cortical neurons, glial cells, and cerebellar neurons.

Gene Symbol	motor neurons	cortical neurons	glial cells	cerebellar neurons
Downregulated genes				
Fus	0.31	0.18	0.47	0.46
Cybrd1	0.53	0.5	0.77	0.89
Elmod2	0.6	0.44	0.9	0.93
Tmem44	0.6	0.6	0.82	0.97
Bmp7	0.61	0.69	0.8	0.97
Ostm1	0.62	0.49	0.76	0.91
Fktn	0.62	0.51	0.82	1.06
Ldlrad3	0.64	0.63	0.79	0.98
Klhl5	0.65	0.39	0.87	0.97
Mboat1	0.67	0.64	0.78	0.96
Upregulated genes				
Olfr920	2.52	2.02	2.08	1.17
Dhcr24	2.21	2.46	1.42	1.1
Qpctl	2.14	2.59	1.82	1.13
Smarcd1	2.07	2.66	1.65	1.08
D8Ertd738e	2.02	2.67	1.79	1.15
Rab15	1.94	2.19	1.16	1.04
Capn5	1.92	2.08	1.29	1.04
Mesdc2	1.84	1.96	1.47	1.03
Syt17	1.82	1.93	1.28	1.03
Taf9b	1.82	3.53	1.25	1.14

Table S4. Primer sequences

Gene Symbol	Forward primer	Reverse primer
Real-time qPCR primer		
Fus	GGCTACTCCCAACAGAGCAG	GCTGTTTTGGGTCTGTCCAT
β-actin	GCAAGTGCTTCTAGGCGGAC	AAGAAAGGGTGTAAAACGCAGC
RT-PCR primer		
Synj1	GCGCCTGCTAGAAAAGAATTT	TAGCCGCACCGTATCCAG
Rims1-all	CGACGTGTGGAATCTGTCAT	TTGTTCGATCGCAGAGACAC
Rims1-Ex2-4	CTGCCTGCAAAACACCAAG	GGCTTCCTCTCCGATTTTTC
Scn8a-all	GACCCGTGGAACTGGTTAGA	AAGTTTATGGGCCACACGAC
Scn8a-Ex4a	GCAATGTTTCAGCTCTACGC	AAGTTTATGGGCCACACGAC
Stxbp1	TGTGAGCCTGAATGAGATGC	AGCAGTTTCTGTGGGGTGAG
Kcnip1-all	GTTTTTCCCTCACGGAGATG	CCTGGGAGTGTCCTCTTTGA
Kcnip1-Ex2	ACATCGCCTGGTGGTATTACC	GGTGTCGAAGGCATTGAAGA
Fmr1	GCCACCAAGTTCCCTACCTT	TGCTGTCTTTTGTTCCCACA
Abi1	TTCCCAGTATGGCACAATGA	GGCGGAGAGTCATCAAACAT
CamK2a	CGGAGGAAACAAGAAGAACG	ATTTCCTGTTTGCGCACTTT
Cttn	AGCTGCATGAATCCCAAAAA	ACTGGTTTTGCTGGTTACGG
Grip1	CCAGGTCAAGCTGGAGATTC	CTGGTGGAGTAGAGGCTTCG
Nav2	GCCAGTTTCACATCAGCAAG	GGAGAGGTGACAGGTTTGGA
Neo1	GATGCCCTTTGACTCTCAGC	TCAGGGTCCTGATGGTCAGT
Ndrg3	CTCACCGAGGCCTTCAAGTA	CTTTCTCCAGAGCCAATGCT
Rapgef4	ATGGAAACGGGCTCTAACAA	TCAGTCCCAACACAGCATTG
Sh3kbp1	CTGAAAGTCGGGGACATCAT	GAGTCGCCTCCATCACTCTC
Slc1a2	ACCGAATGCAGGAAGACATC	GGCTGAGAATCGGGTCATTA
Ttll5	AAACACCCTGCCACAAAAAG	GCACAGCAGGTTCTTCTTCC
Zhx1	TCGCTCTGAAATGCAAGATG	CACTGCTGAACTGCTGAAATG
Braf	CGACCAGCAGATGAAGATCA	TCCGAGGATGAGGAAGAAGA
Mapt	TCCCCCTAAGTCACCATCAG	GCCAATCTTCGACTGGACTC
Dlgap1	GTGAGGACCATCCAGAGCAG	GGACTGGAGGTGGTGTCTTC
Anks1b	TGGCATCTTTGGGAGATAGG	TTCATTAGGAGGTCGCAAGG
Brcc3-all	CGGCGTTCTGACAAGAGAA	CCAACCTTTCTGCCTCTGTT
Brcc3-3'UTR	ATGGATCCTGCATCTGACCT	GGGAGGGACAGTCTGGAAAT
Tia1	TGAAAGTGAATTGGGCAACA	GGGCATCTGAAATTCTTCCA
Caskin1	CATGCAGGCTCTGAAGGAGT	TGTCCACCAGCACCTTGTAA
Clec16a	CTGAAGATGCCCAGGAAGAC	GTGTTCTGCTCCTGCACTGA
Elmo2	TCCAACCAGGAGATCCAGAC	TCAGGATTATGGACCGAAGG
Erc2	GCATCCTCGTTAGCTTCTGC	ATCTCCAGCAACCTGTCCAC
Fkbp15-all	TCGAATGGCAGTCAACAAAG	CCGACATGCTAGGAAGAAGC
Fkbp15-5'UTR	TGCCATCTTTGGGAAGAGAC	CCTGTGAGAACCTGGGGTTA
Grm5	GCACGTAGGAGATGGCAAGT	GGGTGCTCTTCTCATTCTGG
Lrrc7	AGAGGAGAGCTGTGGCAAAA	CCATTGGCCACTAGGAGAGA
Pdzd4	CTCTTCCCGCTCTCCTCTTT	AGTGTTCTCCTCCTGGCTCA
Tcerg1l-001	ATCACAGTGACAACCGGACA	CTTCTCCTGGTGGGGATACA

Tcerg1l-003	TGCAGGGACAGTGCAGATAG	TCATGTGTGGGCCTTTGTTA
Xpr1-all	TCAAGATGGACTGGGGTCTC	TTGGATAGTCCAAGCGAAGC
Xpr1-Ex13	CCAACCCATGAGGTCAGACT	ACTGCCCAGCAAATTGATTC
Anks1	CCTCCAAACCAGTGCCTAAG	GCCTCTTGGTCAGAATCAGG
Wdr35	TCGTCCCGAATACTGTGTTG	GGGTGTGCCAATAGAATTGC
Sgce	CGACCACTGGCACATTCTTA	CAGGCTTTGGATAAGGTGGA
Fip1l1	GGATACGAATGGGACTGGAA	TGGCAGACCAGTCTTGAACA
Fxr1	TGCTGTTCTGATGGATGGAC	GAAGCGCTAGTTGGACCATT
Tsc22d2	ATGAACAGTCTGGCCACCTC	TTCACCAGATCCATTGCTTG
Map4	CCAAGGTCGGCTCTACAGAA	CACCAGGCTTGTGCTTGATA
RNA imunoprecipitation primer		
Mapt Ex6	TCCCCCTAAGTCACCATCAG	GCCAATCTTCGACCTGACAT
Dlgap1 Ex11	ATCACAGCCCAGAGTAGCAC	GACTTTCGGTGGAGTTGCTG
Stxbp1 Ex20	GCTCCCTTCCCAAAATGCTC	GAAGAACTCAGCACGGCAAC
Gapdh	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG
intergenic primers	GGCTTGAGGCAGACTACAGG	TGGTCAATGAGCCCACATAA

Semi-quantitative RT-PCR primer

Gene Symbol	Forward primer	Reverse primer	Taqman probe
Snap25-001	AGGAAGGGATGGACCAAATC	AAGCCCGCAGAATTTTCCTA	CATGAAAGAAGCAGAAAAGAATTTGACGG
Snap25-002	CAACTCGATCGTGTCGAAGA	CATATGAAAAGGCCACAGCA	ACCATATCAACCAAGACATGAAGGAGGC
Ntng1-205	GCCGAATATTTCTTCCCTTG	GCTTGTGGTTTGCAACTTGA	CCCAAAACAAGTTGCTCCCAAATTAGC
Ntng1-others	AGCACACGGTCTTGGAAATC	CGAAAAACGCAAACCTGTCT	CTCCACTGGGTACTCCACGAATAGCAA
Smarca1-006	ACCACGGCCACTGAGAAG	TCTTCTGATGAAAGTAGATGAGGTC	CGAGAAGAAGGAGCCAGCTATTCACTT
Smarca1-other	TATGCCCTTGAAAGCAGACC	TACTCGAGGACGAGCCAGTT	TGCACATTTCATTCAGCCTTCAGCA

Supplementary references

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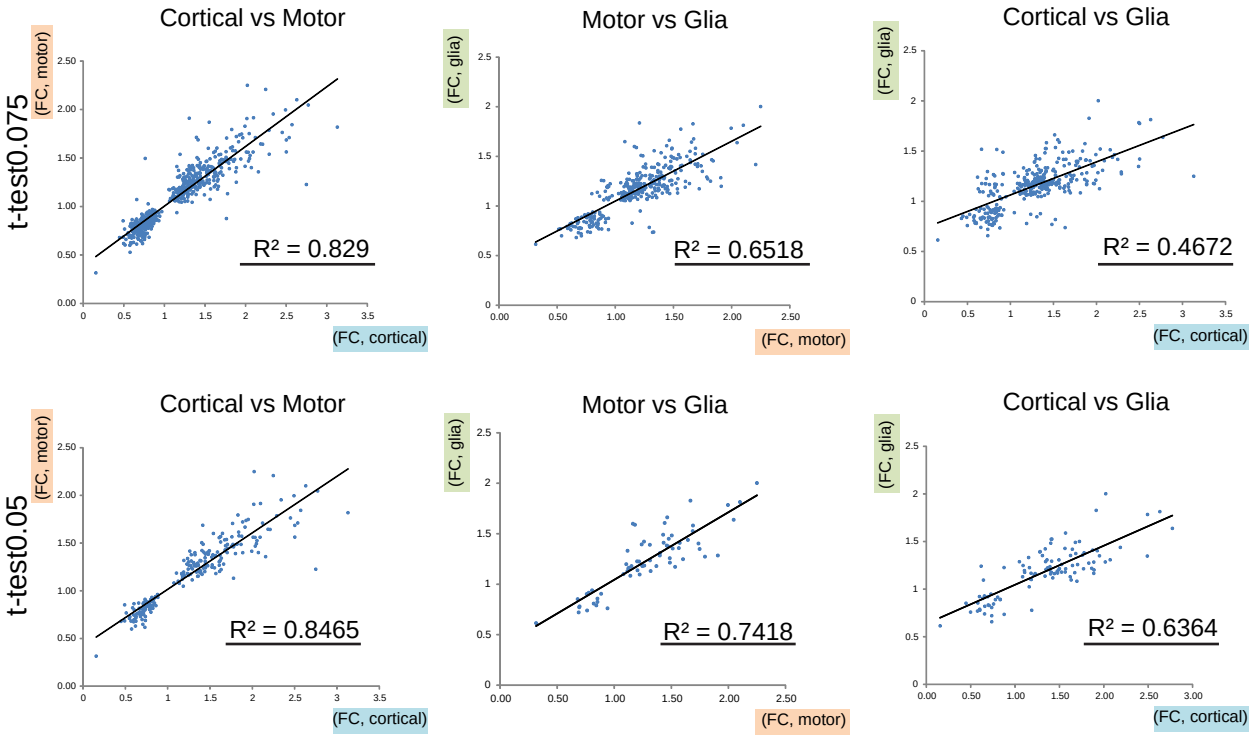
4. Dennis, G., Jr. et al. DAVID: Database for Annotation, Visualization, and Integrated Discovery. *Genome Biol* **4**, P3 (2003).

Supplementary Figure S2

A



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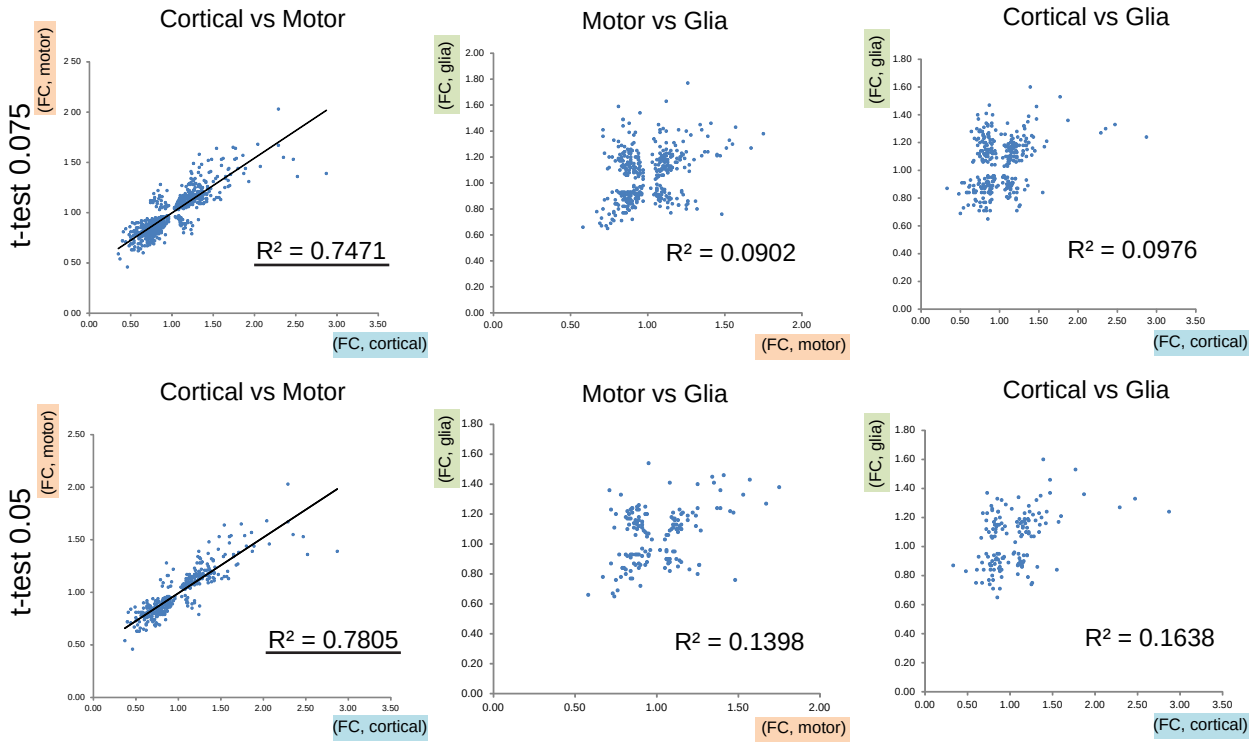

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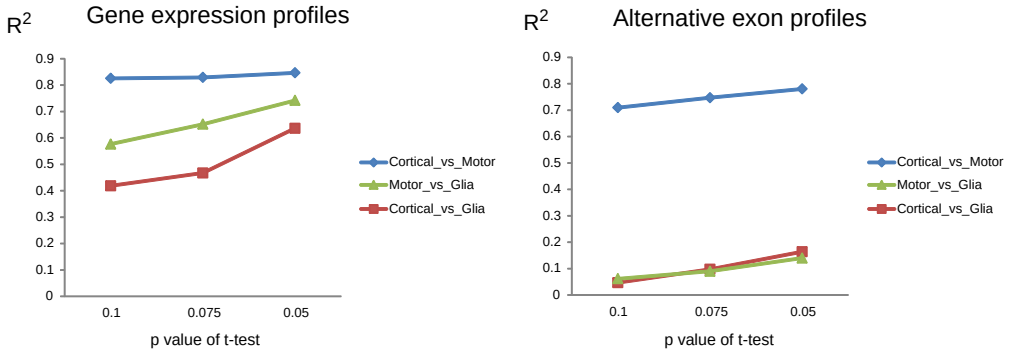
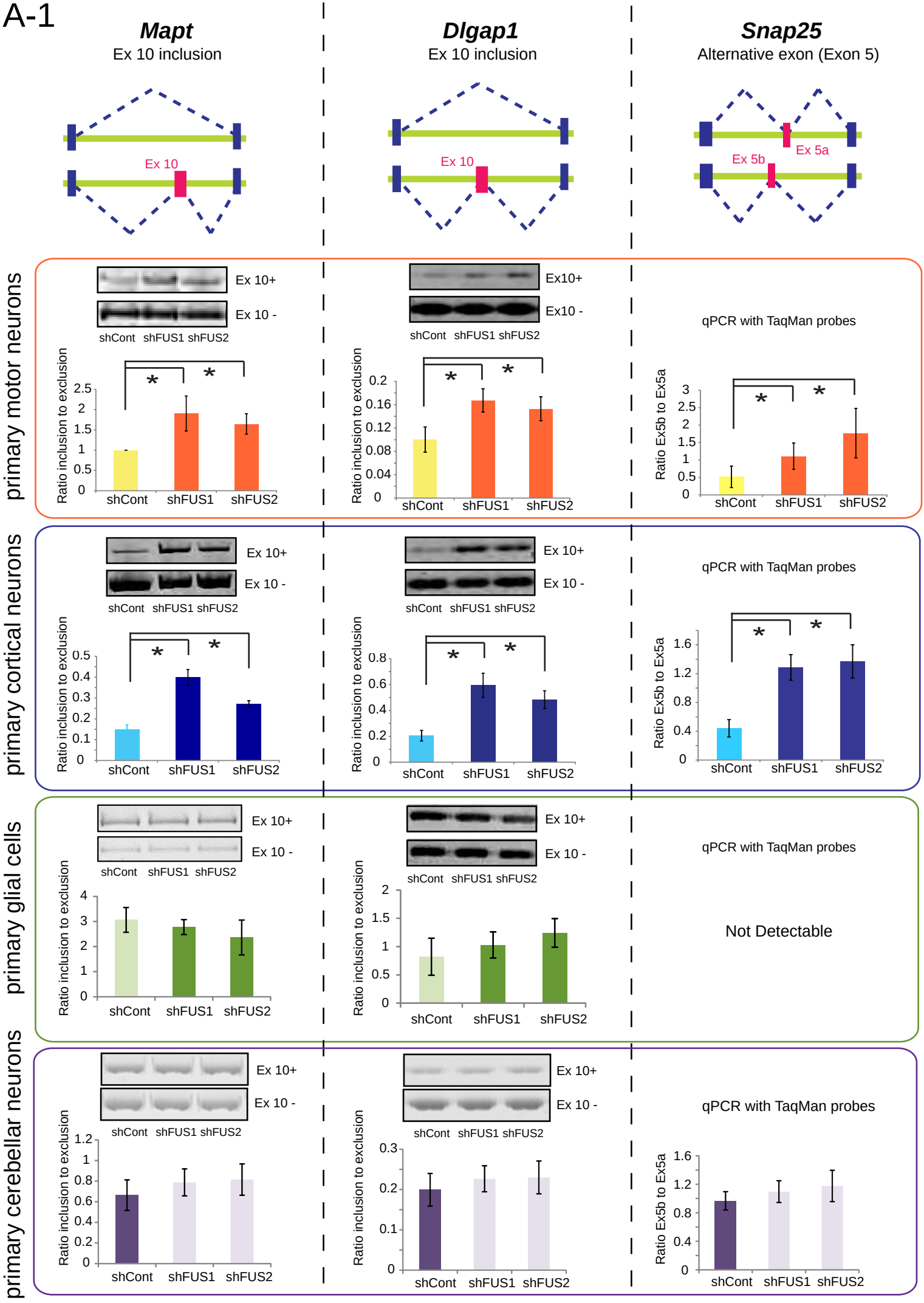


Supplementary Figure S3

A-1



Supplementary Figure S3

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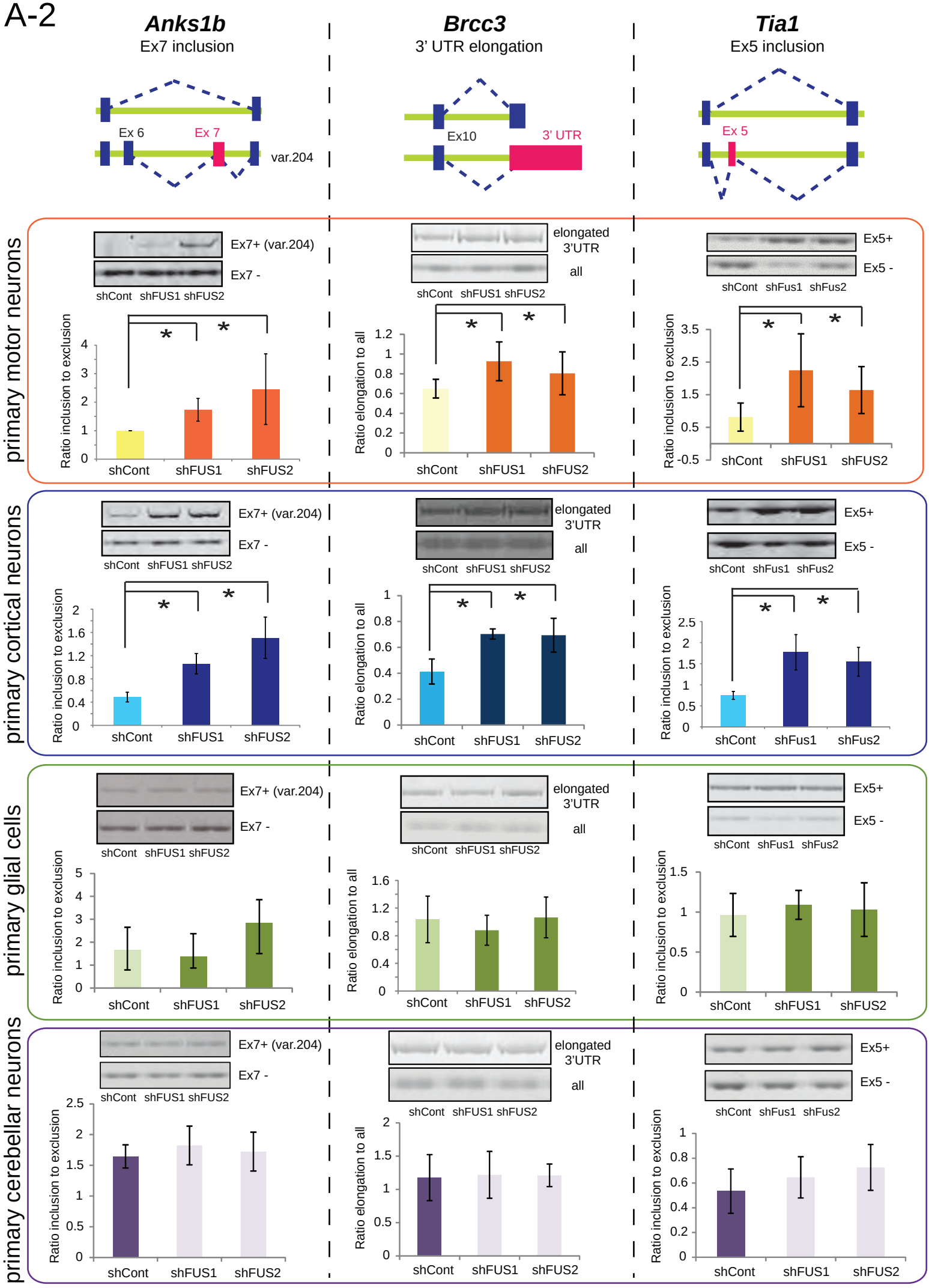

Supplementary Figure S3

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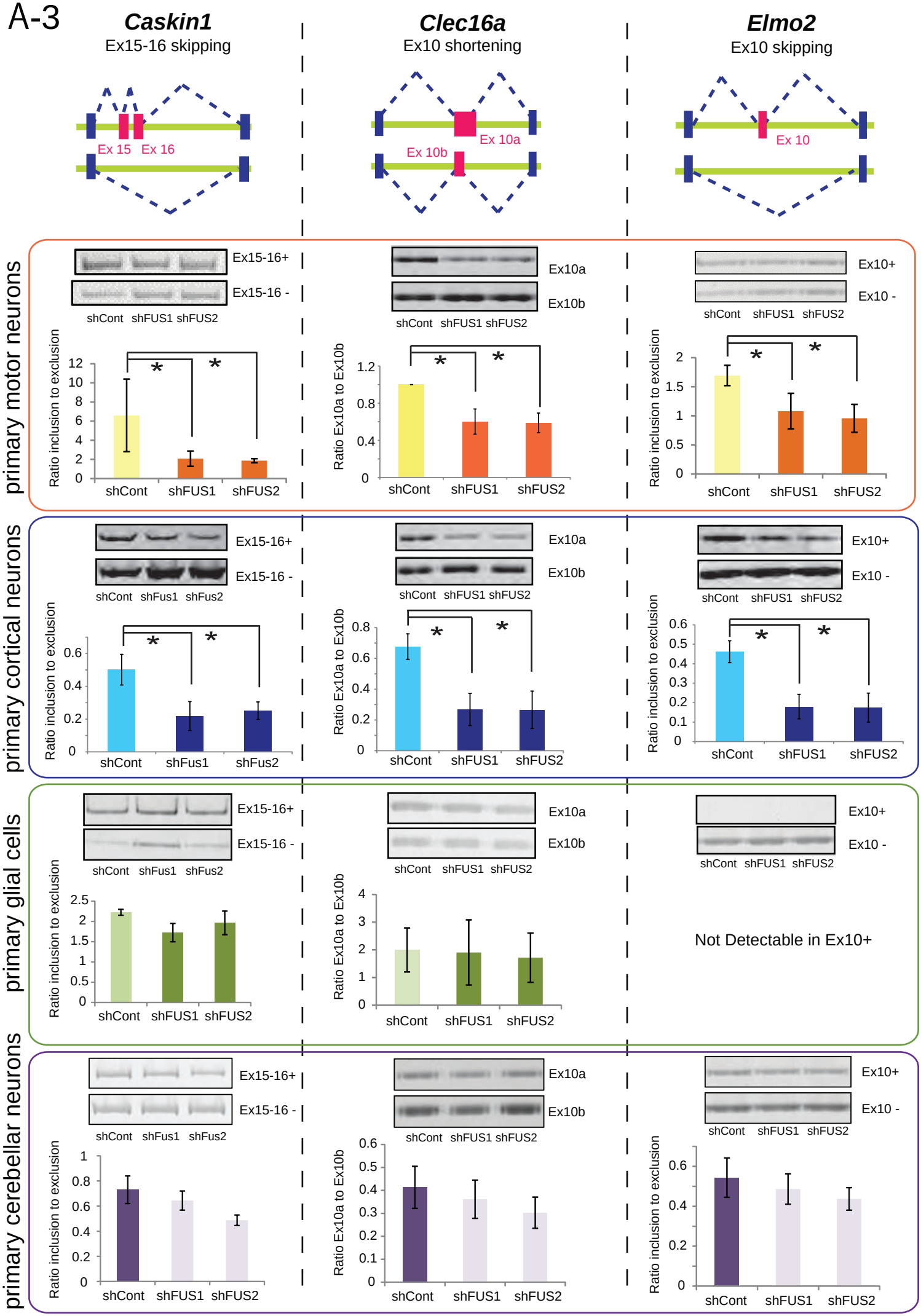

Supplementary Figure S3

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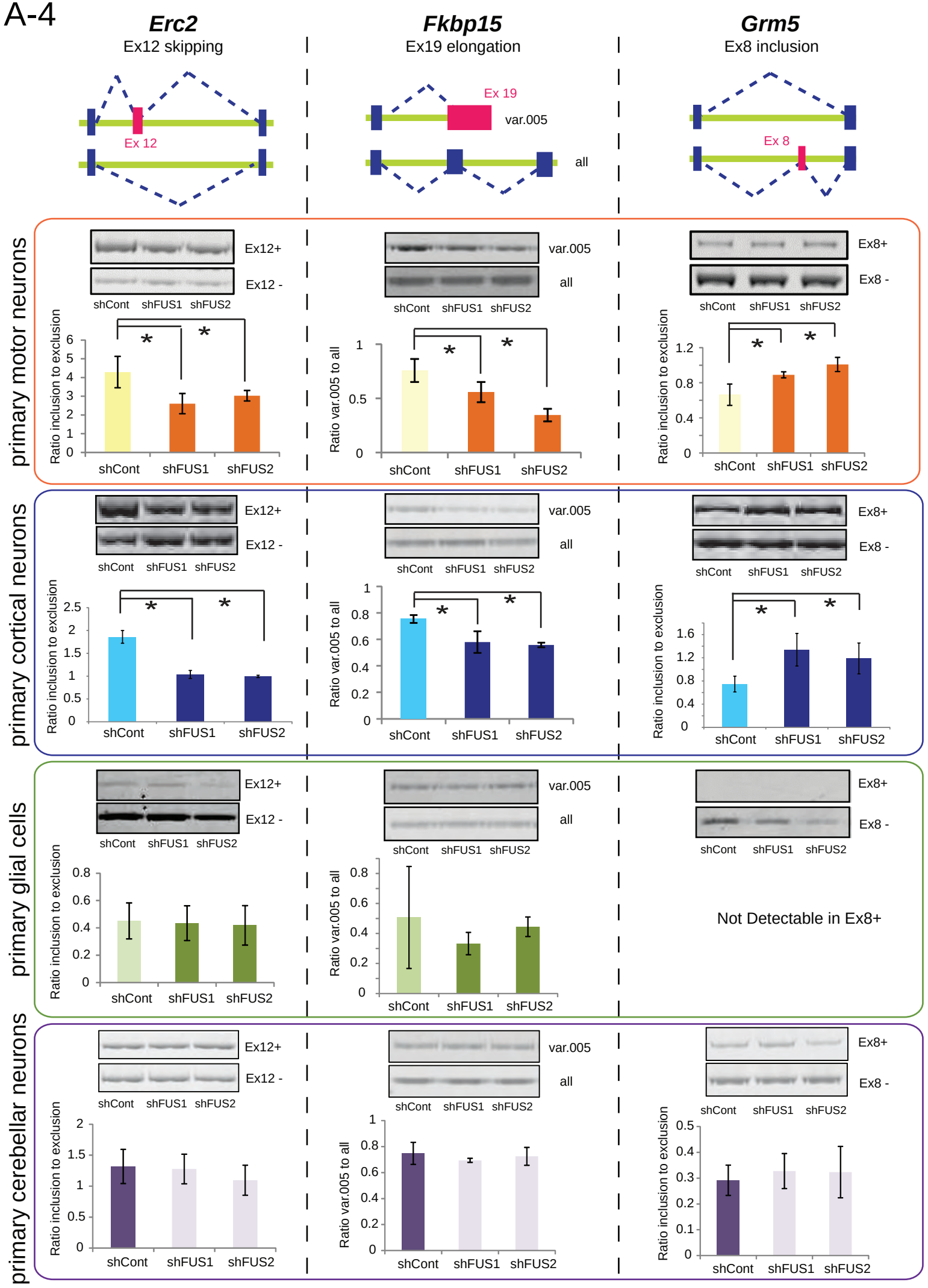

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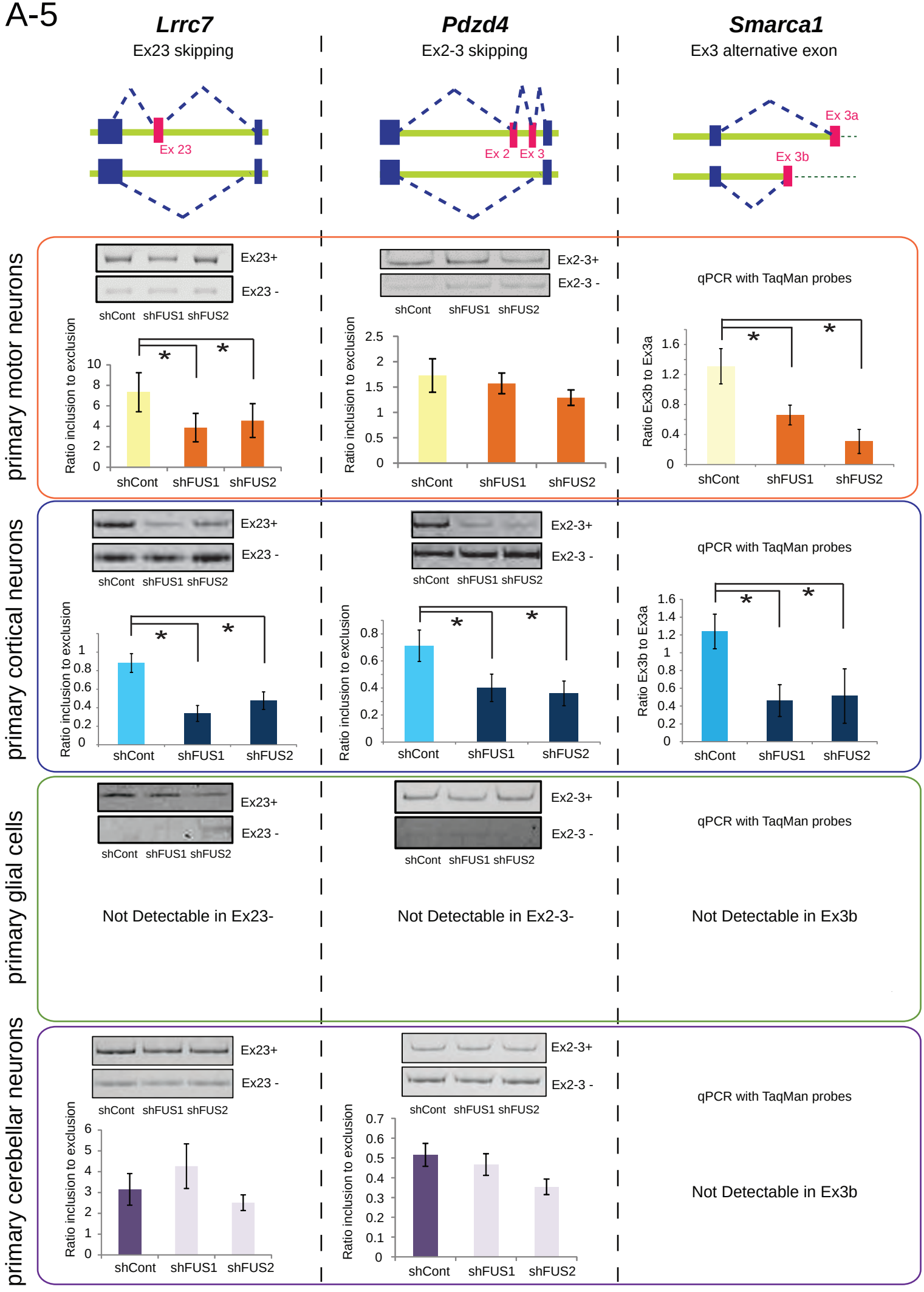

Supplementary Figure S3
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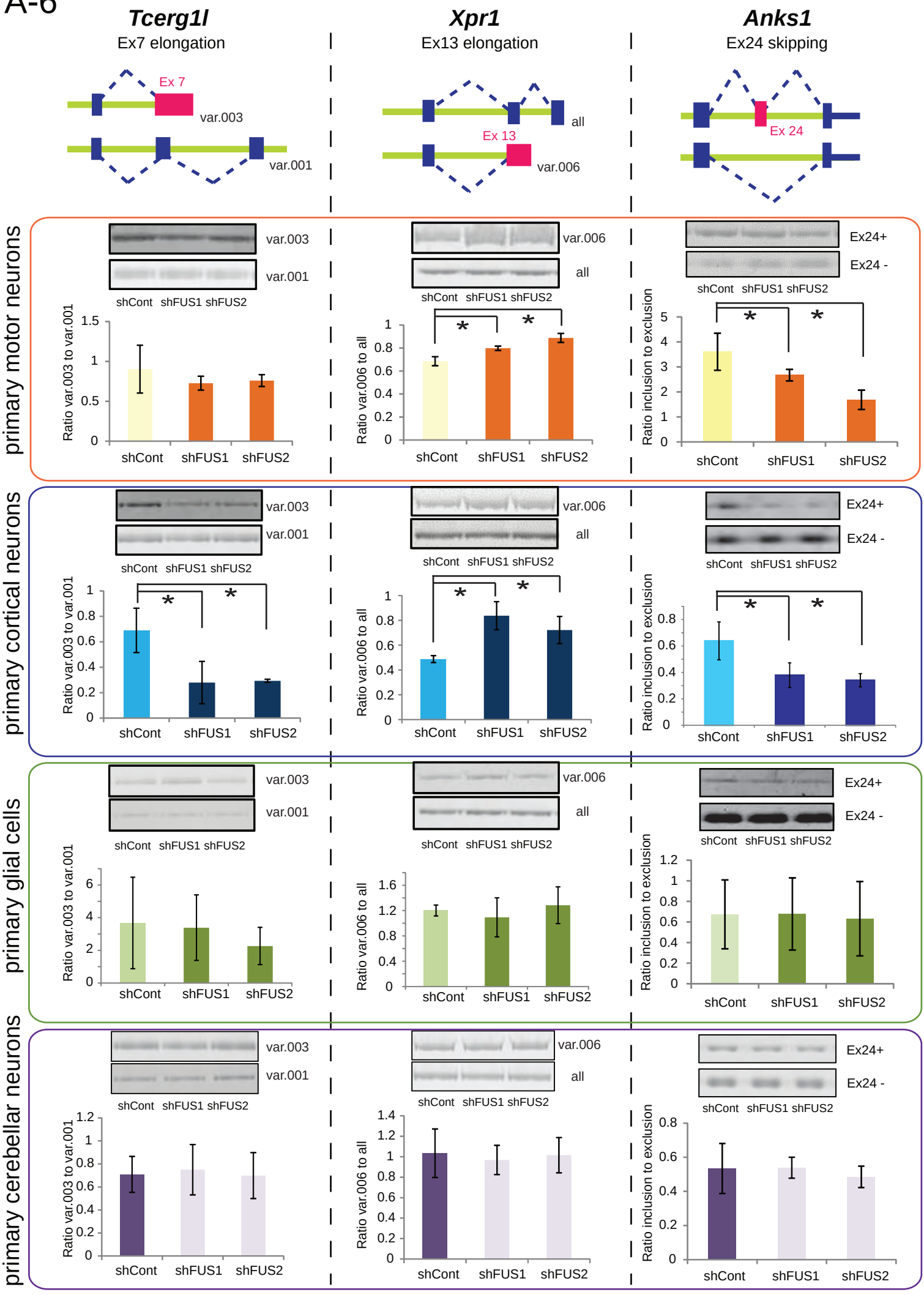

Supplementary Figure S3

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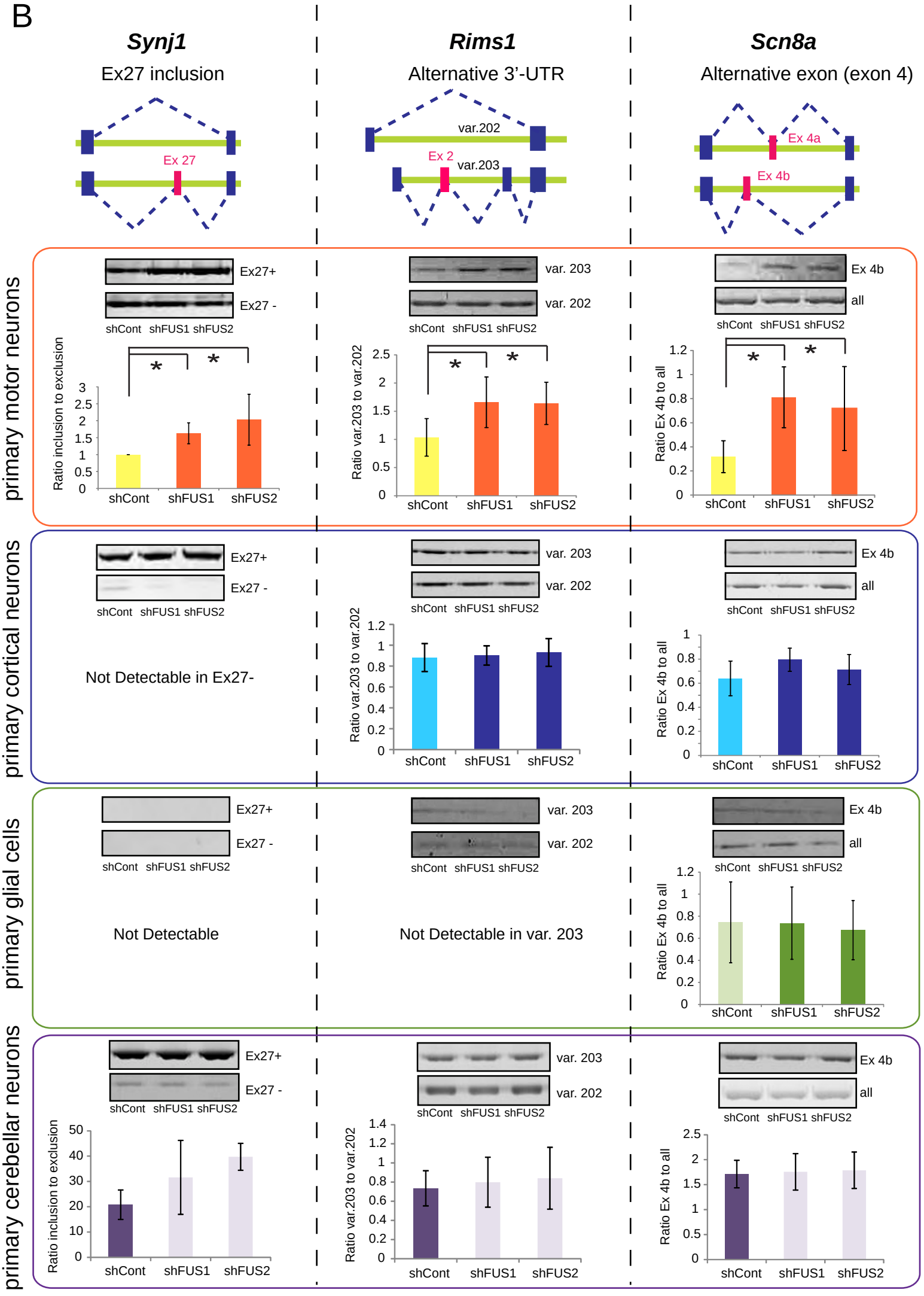
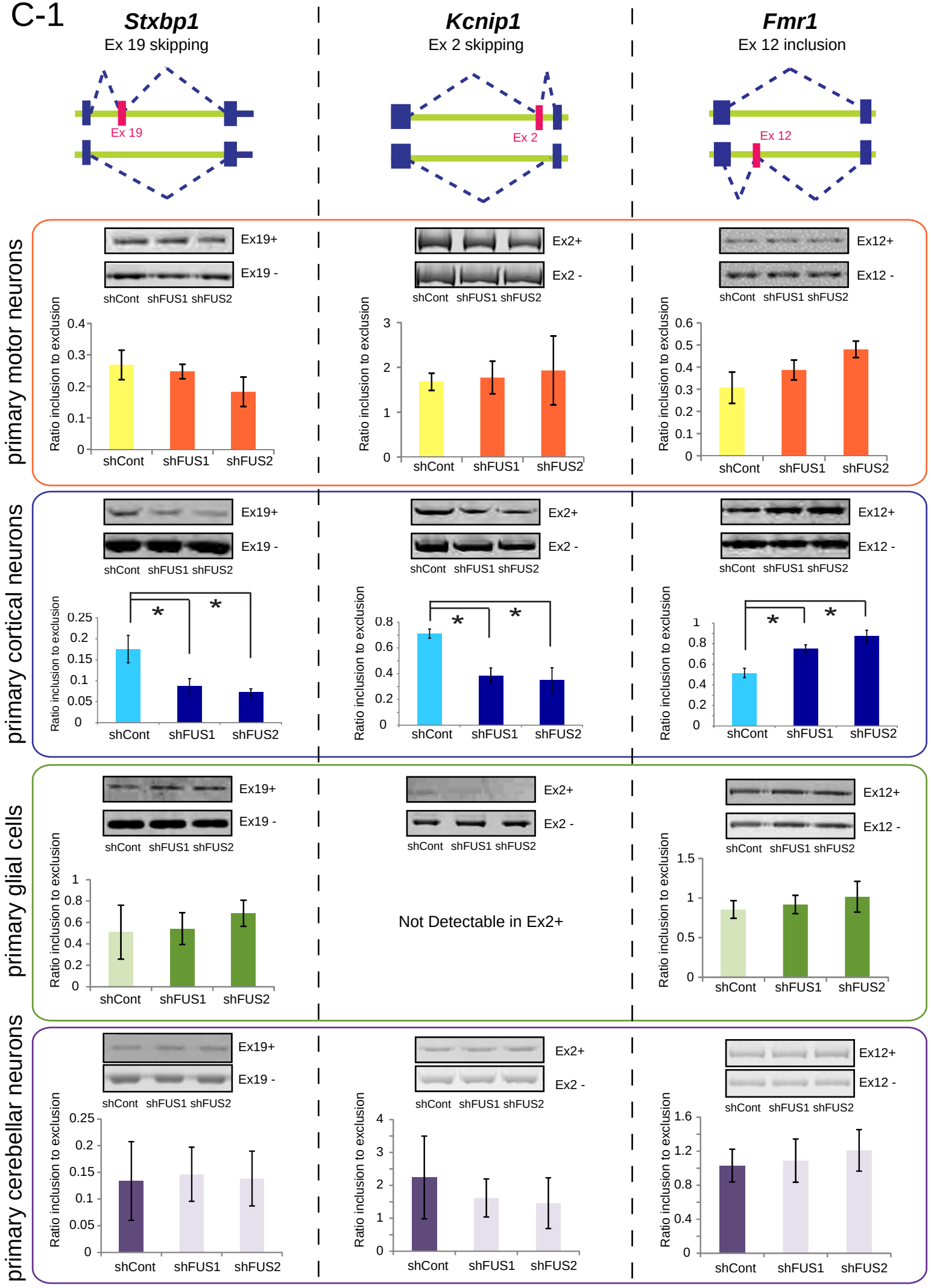
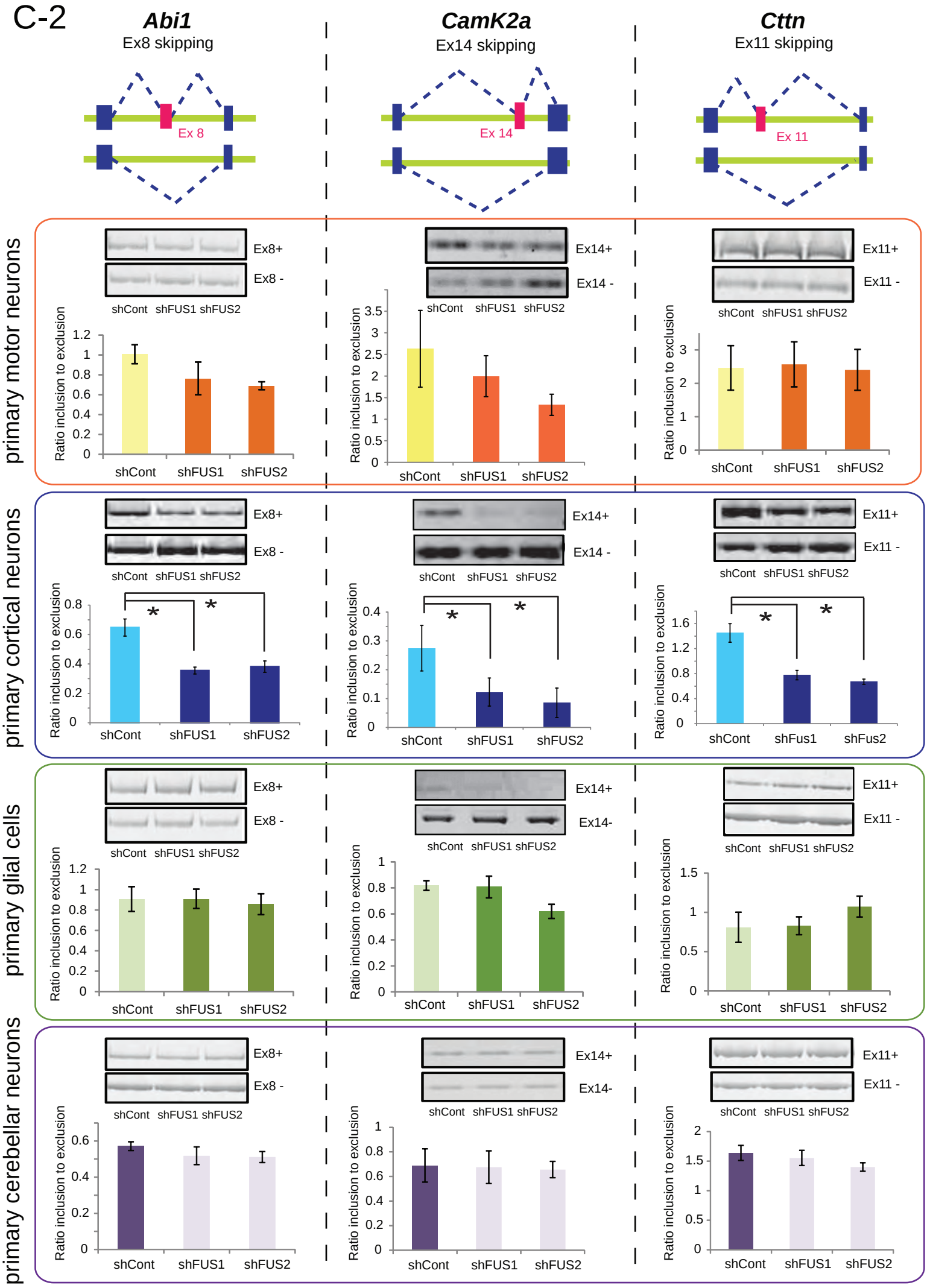


Supplementary Figure S3

C-1

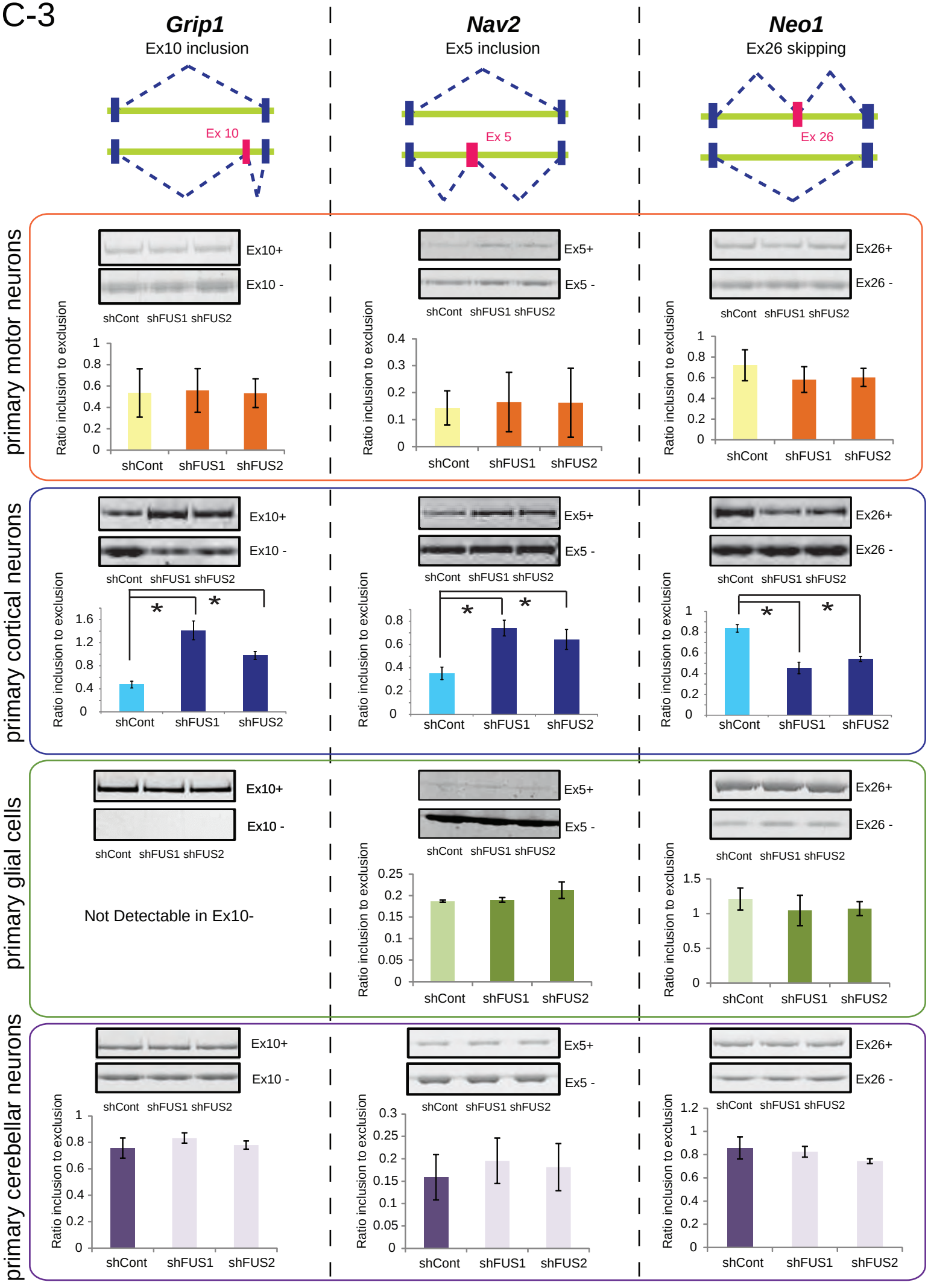


Supplementary Figure S3



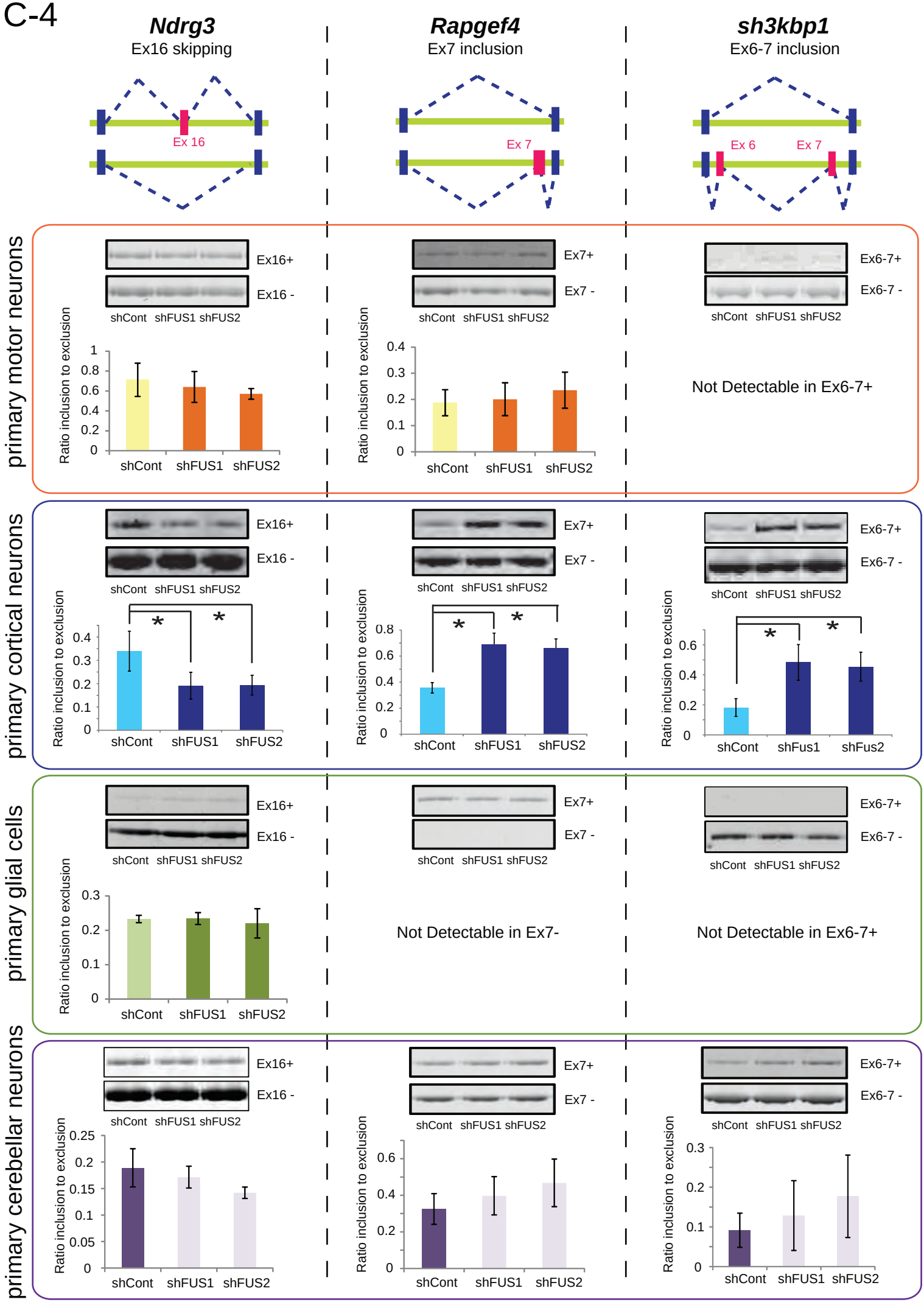
Supplementary Figure S3

C-3



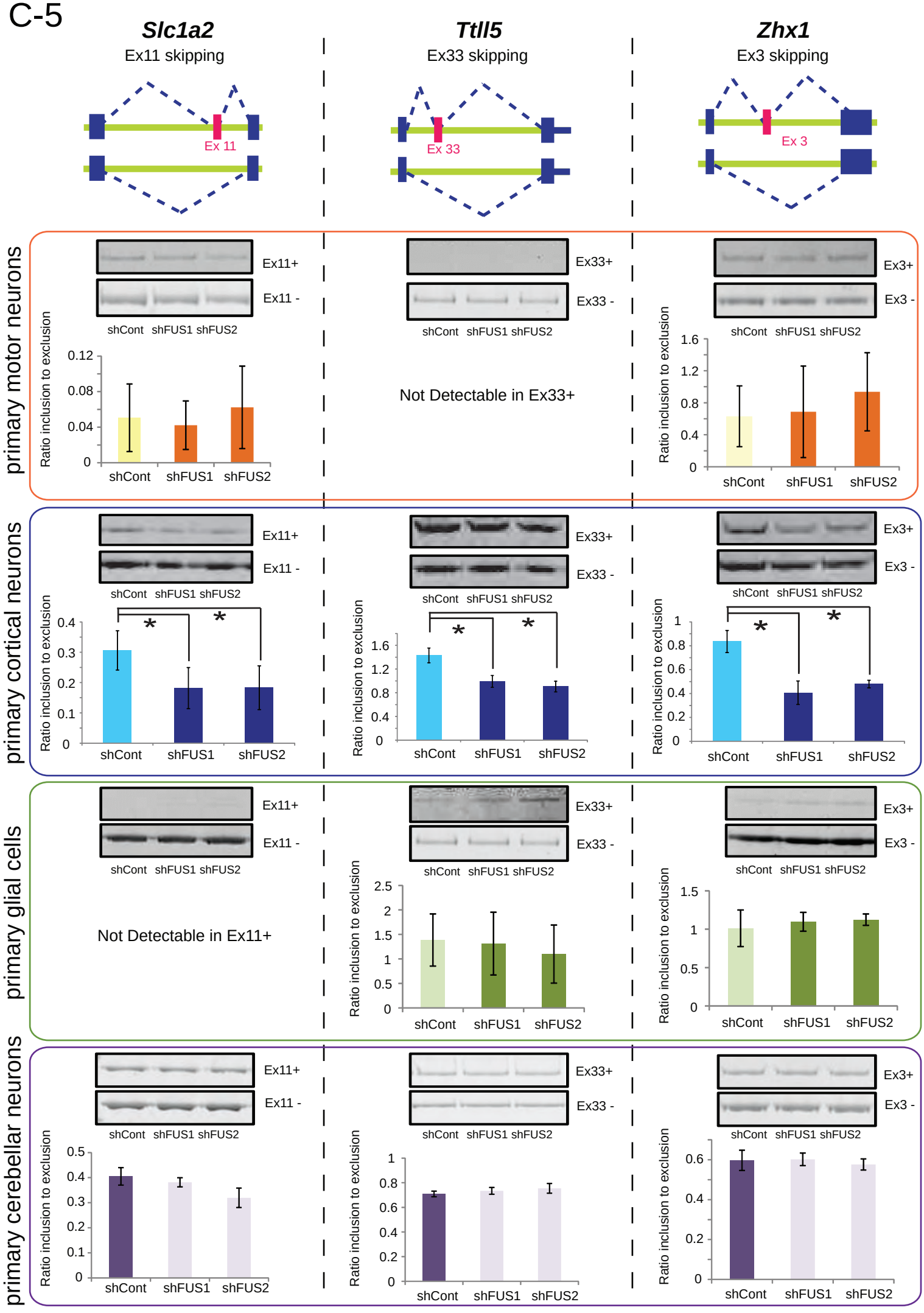
Supplementary Figure S3

C-4



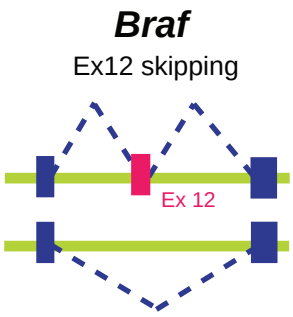
Supplementary Figure S3

C-5

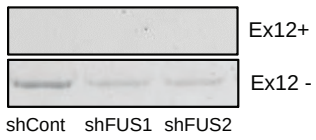


Supplementary Figure S3

C-6

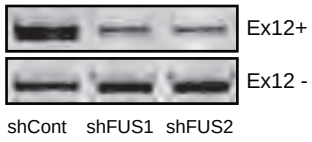


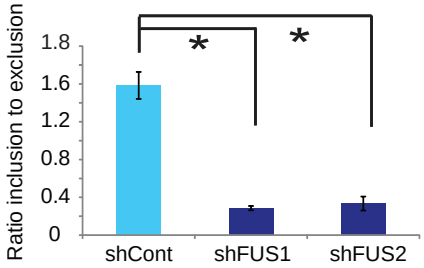
primary motor neurons



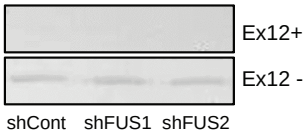
Not Detectable in Ex12+

primary cortical neurons



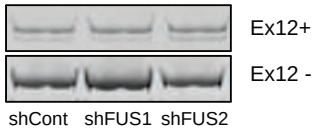


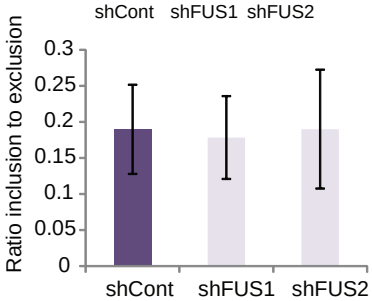
primary glial cells



Not Detectable in Ex12+

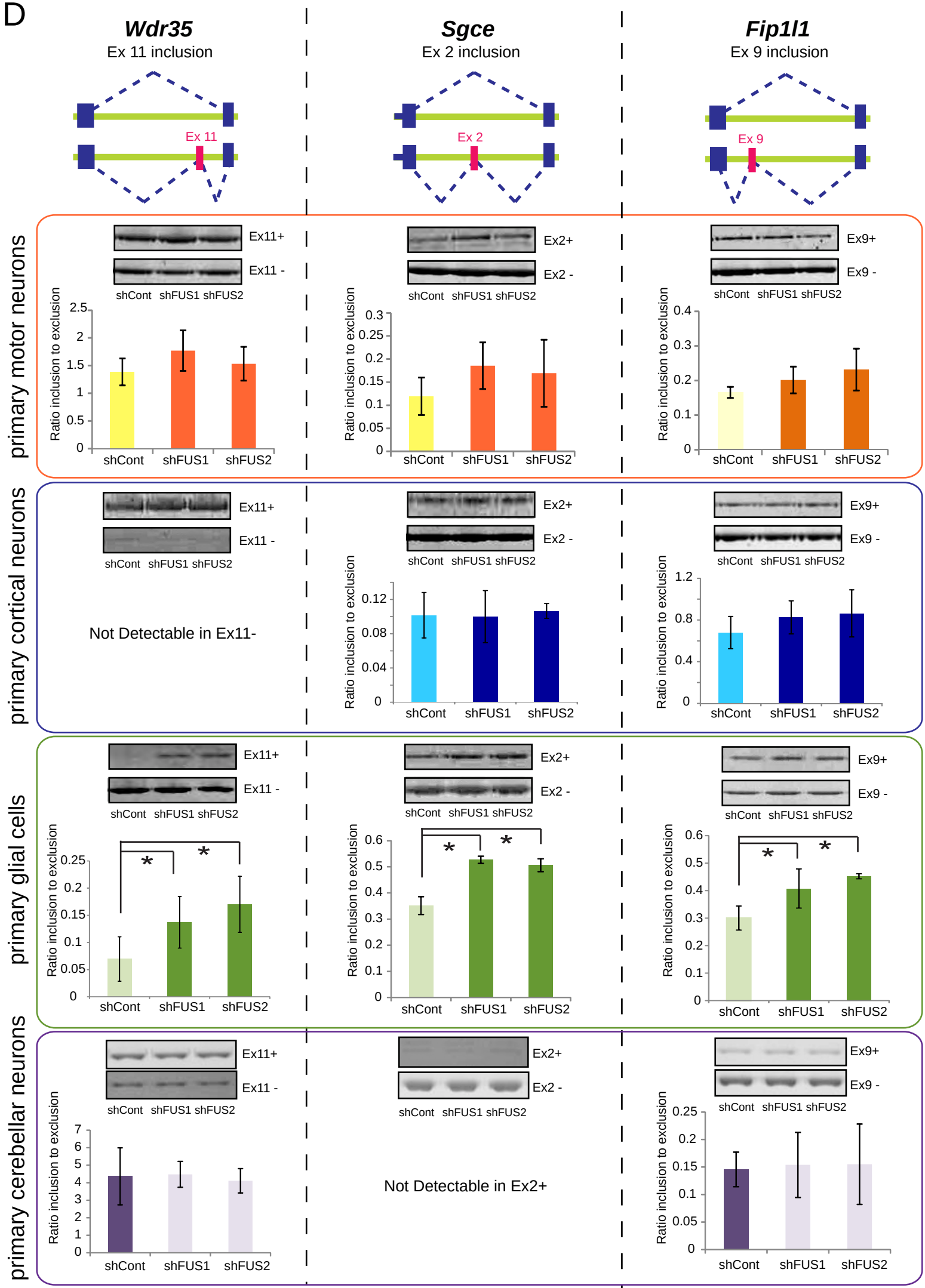
primary cerebellar neurons





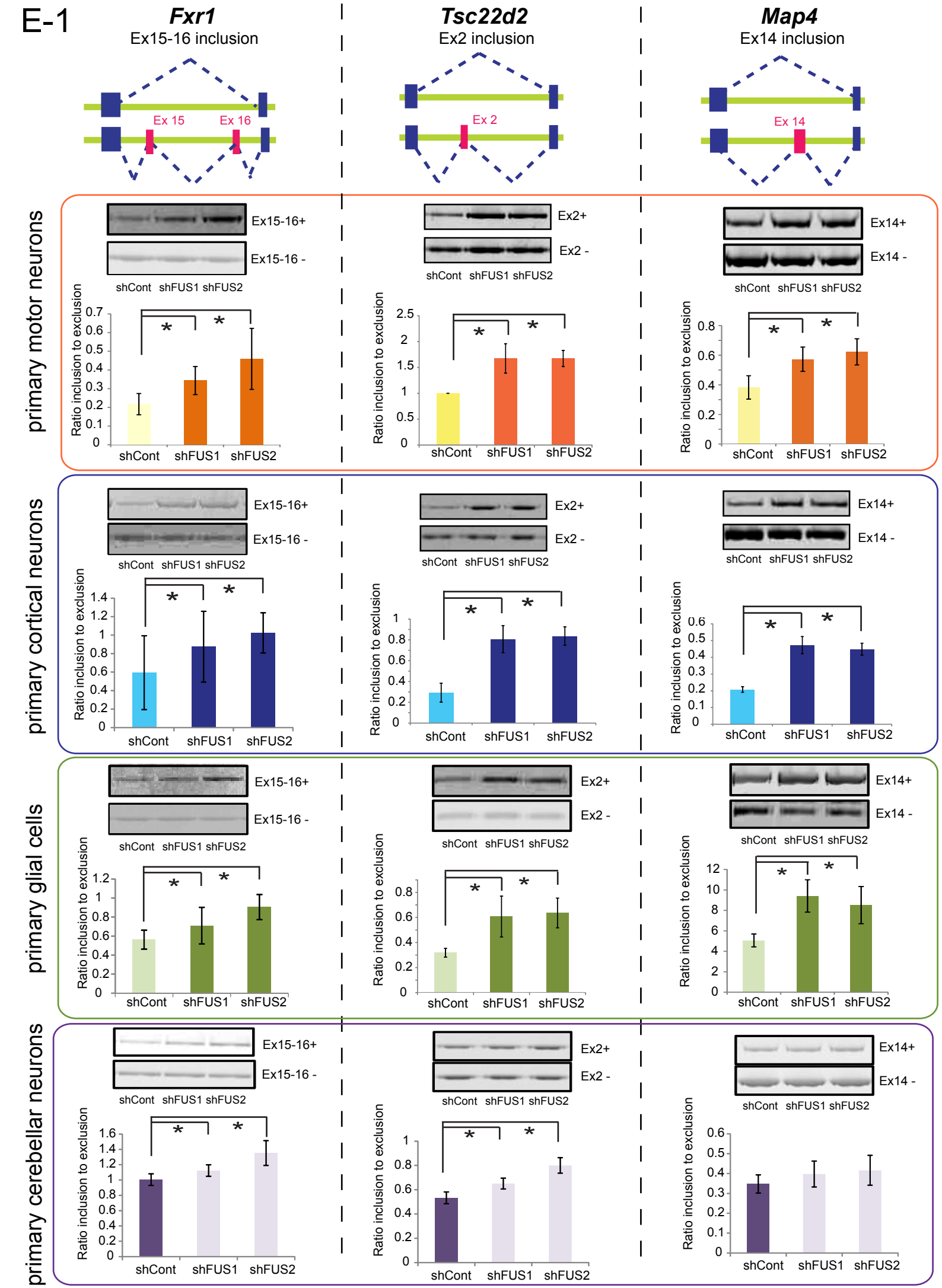
Supplementary Figure S3

D



Supplementary Figure S3

E-1

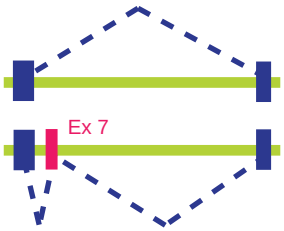


Supplementary Figure S3

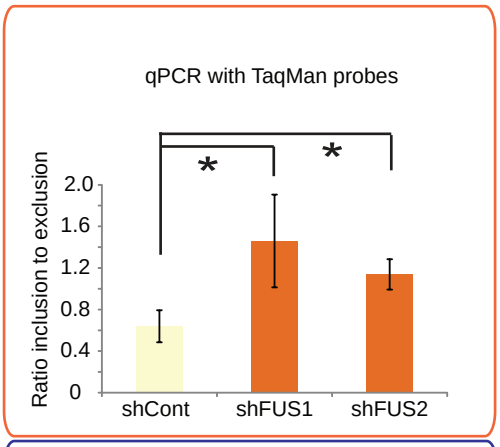
E-2

Ntng1

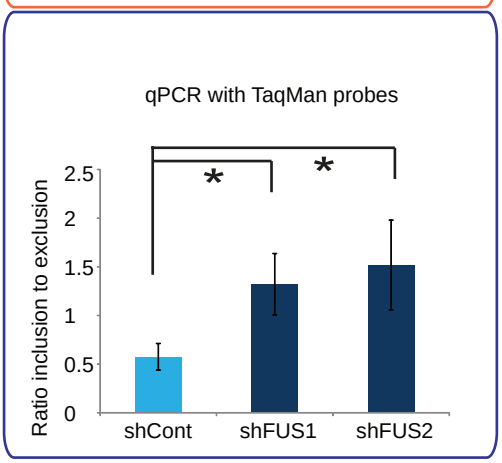
Ex7 inclusion



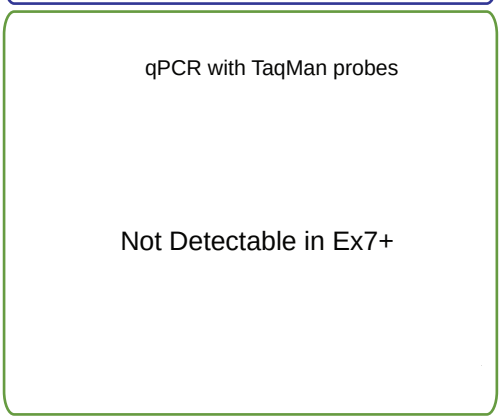
primary motor neurons



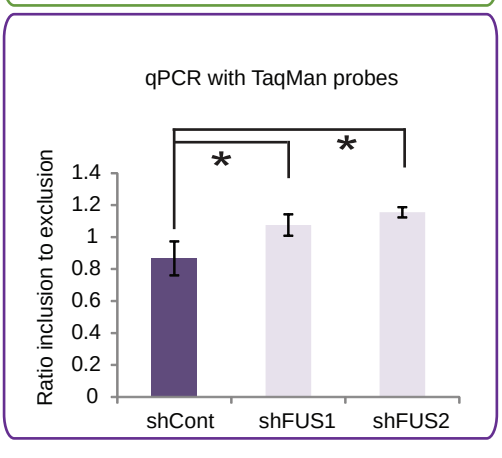
primary cortical neurons



primary glial cells



primary cerebellar neurons



Supplementary Figure S4