PNAS
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Supplementary Information for

The proteasome regulator PI31 is required for protein homeostasis, synapse maintenance and neuronal survival in mice

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Movies S1 to S3

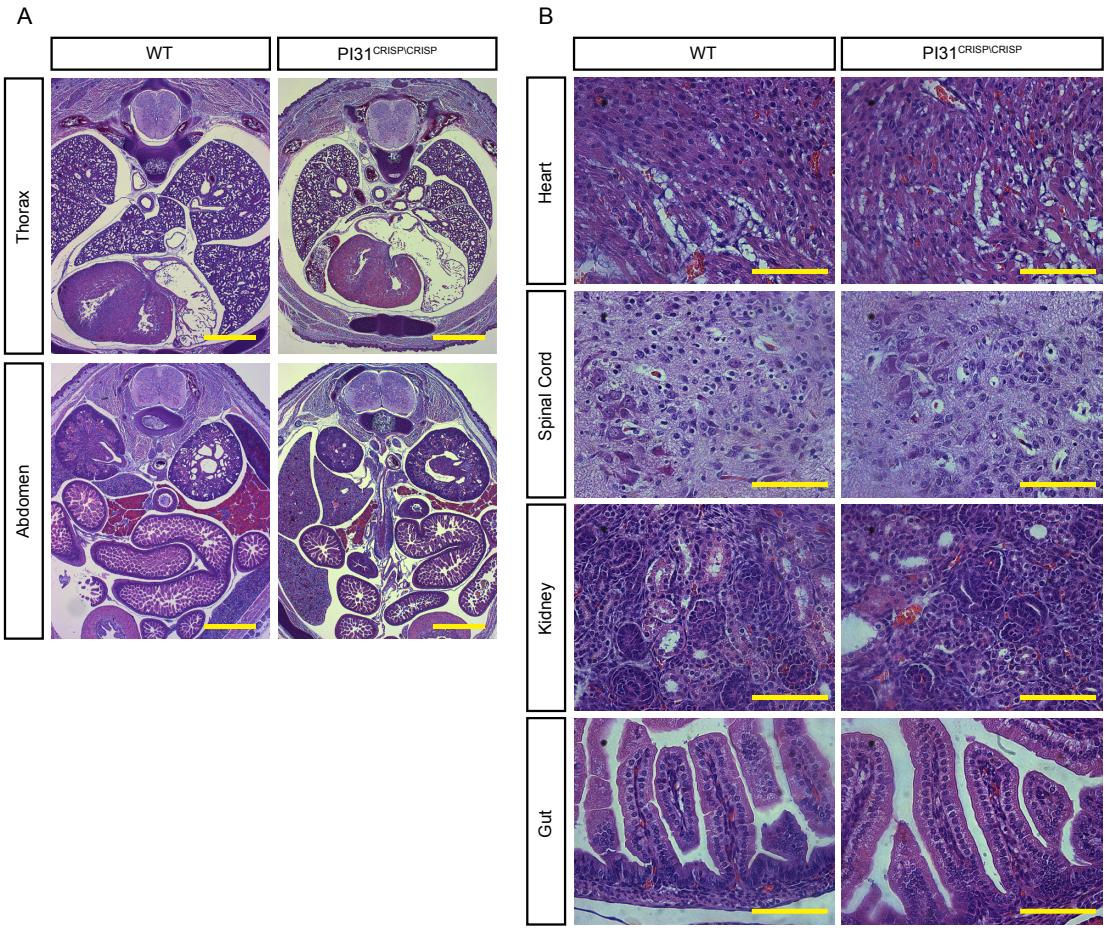

Fig. S1. Histological analysis of E18.5 PI31$^{CRISP\CRISP}$ embryos. Low (A) and high (B) magnification of H&E coronal sections taken from different levels of the apical-caudal axis of E18.5 PI31$^{CRISP\CRISP}$ and control littermates. Loss of PI31 did not have profound consequences for the development of many embryonic tissues and organs. Scale bars are 1mm and 0.01mm for figures in panel A and B respectively.

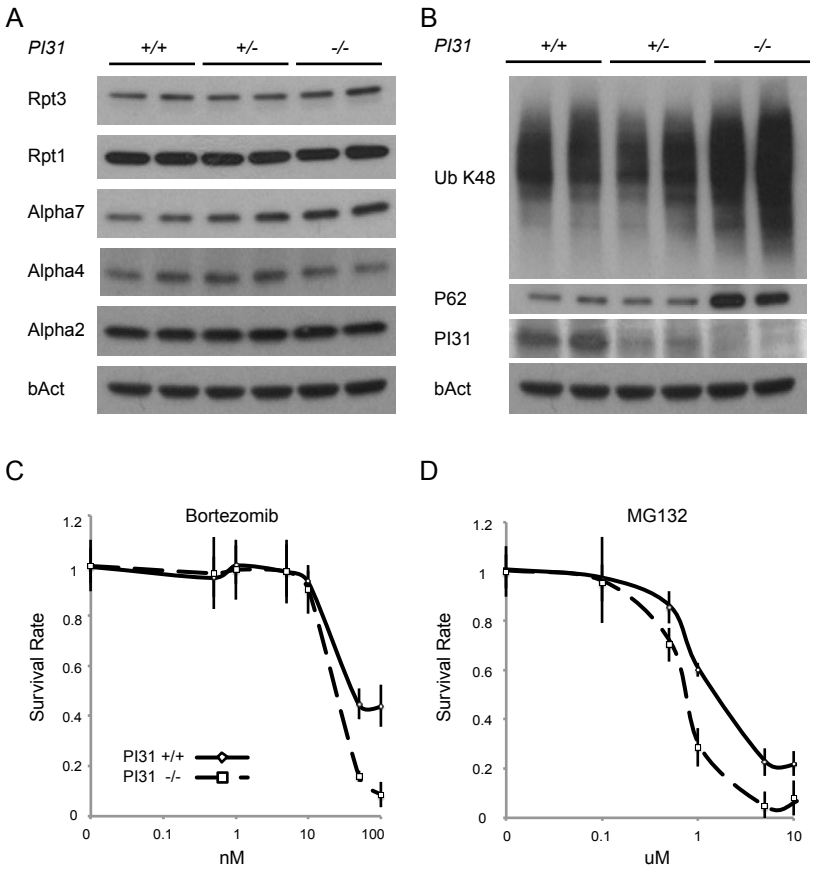

Fig. S2. Loss of PI31 sensitizes mouse embryonic fibroblasts to proteotoxic stress (*A*) Analysis of proteasomal proteins in PI31-Null MEFs. Western blot analysis of WT, heterozygous and PI31 KOF MEFs for individual proteasome subunits does not indicate substantial changes. (*B*) Western blot analysis of WT, Heterozygous and KOF MEFs shows accumulation of poly-ubiquinated proteins (Ub K48) and increased levels of the stress marker p62 in KO MEFs. C, D Diagrams showing survival rate of WT and KOF MEFs treated with Bortezomib (*C*) and MG 132 (*D*) indicated concentrations (average of 3 experiments, each experiment was done in triplicate).

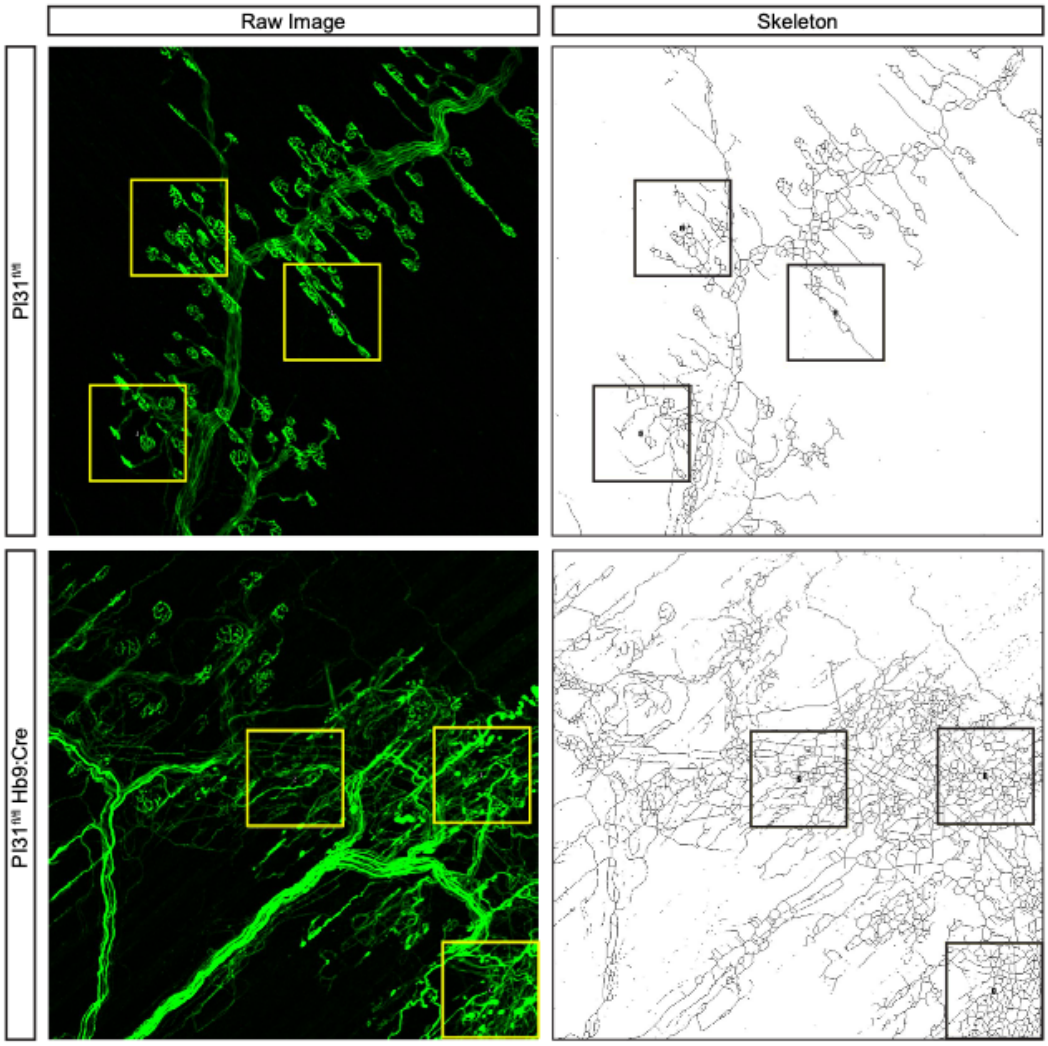

Fig. S3. Quantification of Axonal Sprouting

For each image, 3 Regions Of Interest (ROI) 200x200 pixels were chosen in relevant areas. Using Fiji image analysis software tools, images were then skeletonized. Total axonal length was measured in all ROIs and was subjected to statistical analysis.

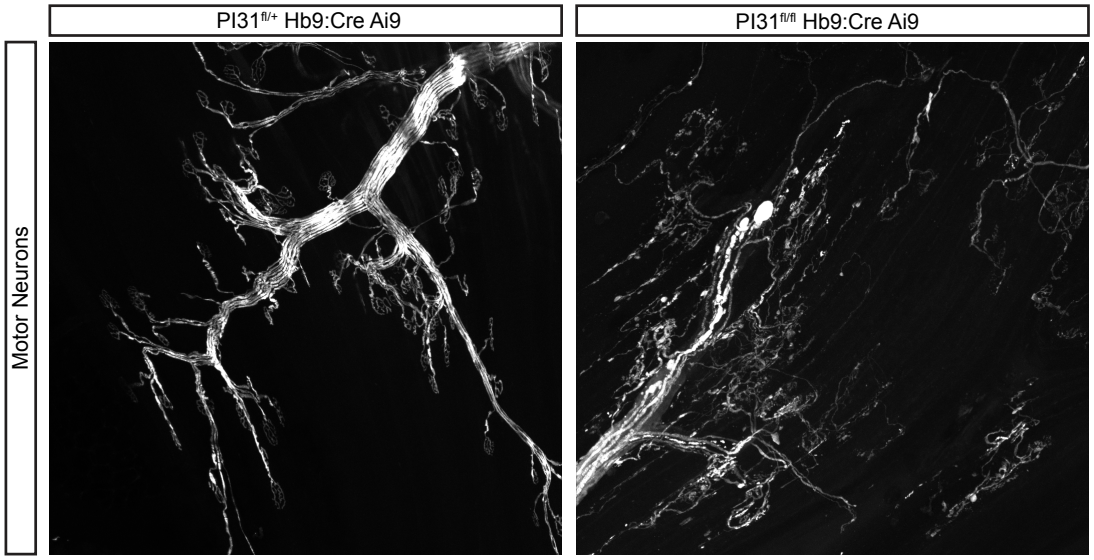

Fig. S4: PI31 Hb9-Cre Heterozygous animals do not show any phenotype

Triangularis Sterni muscle innervation by motor neurons of PI31f/f Hb9-Cre Ai9 and PI31f/+ Hb9-Cre Ai9 mice. Heterozygous PI31 mutants muscle innervation is similar to that of WT. Ai9 mice express tdTomato in a Cre dependent way.

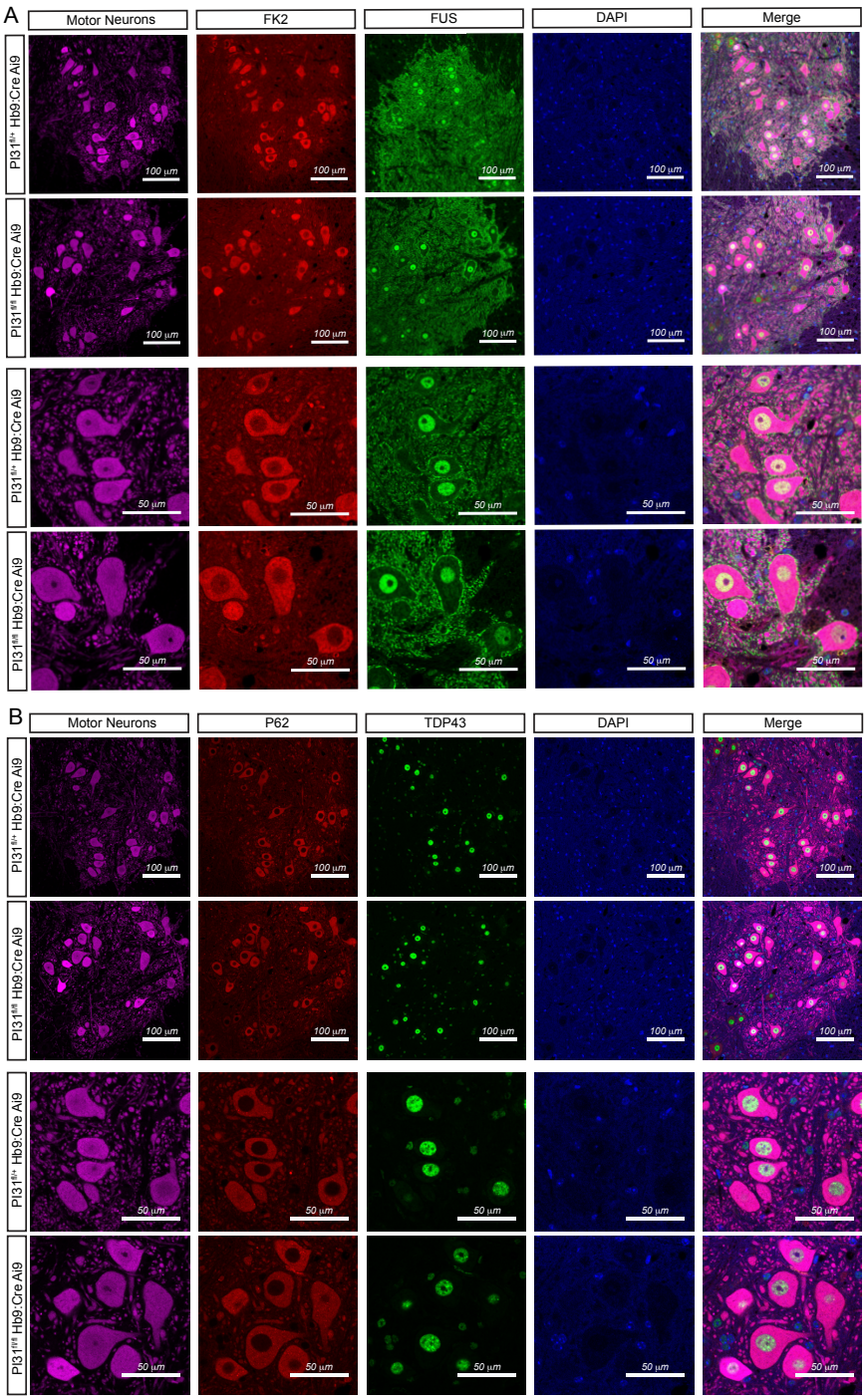

Fig. S5: Pl31-deficient motor neurons do not express proteotoxic stress markers in the soma at 5 months of age.

A, B Immunofluoresence analysis of motor neuron somata did not reveal any significant differences in poly-ubiquitinated proteins (FK2), P62 aggregates or FUS and TDP43 localization between Pl31f/f Hb9-Cre Ai9 and Pl31f/+ Hb9-Cre Ai9 mice. At this age, axonal health was severely impaired.

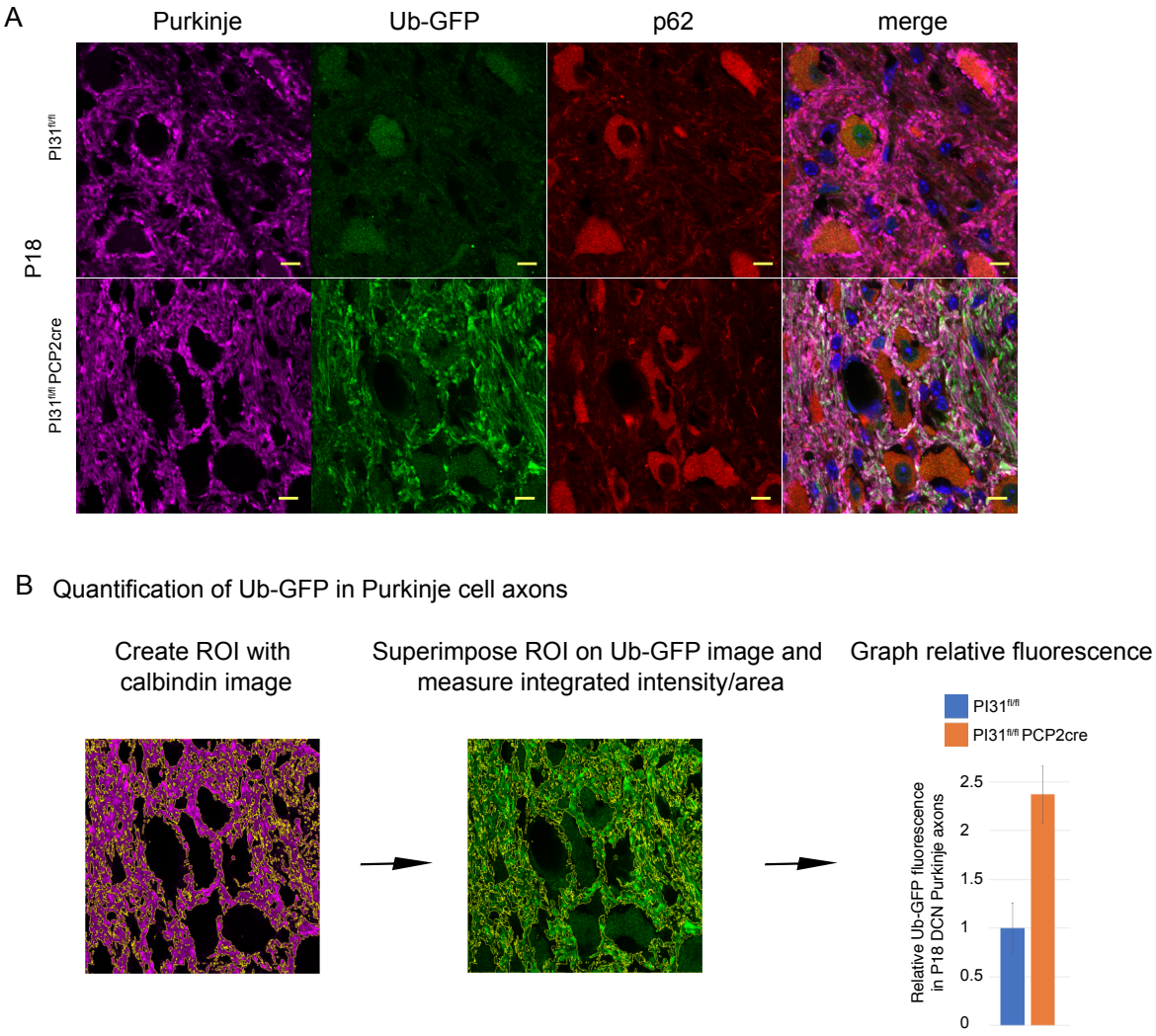

Fig. S6: Loss of PI31 in Purkinje cells leads to an increase of the proteasome substrate Ub-GFP in the DCN of the cerebellum. (*A,B*) P18 cerebellum labeled for Calbindin (magenta) to mark Purkinje cells, Ub-GFP (green), p62 (red), and Hoechst 33342 (blue) for nuclei. *PI31$^{fl/fl}$* and *PI31$^{fl/fl}$ Pcp2-Cre* denote *PI31$^{fl/fl}$ Ub-GFP* and *PI31$^{fl/fl}$ Pcp2-Cre Ub-GFP* mice respectively. (A) By P18, we can see a clear increase of Ub-GFP In Purkinje axons in the DCN of *PI31$^{fl/fl}$ Pcp2-Cre* mice as compared to control *PI31$^{fl/fl}$* littermates. Scale bar 10 µm. (*B*) Illustration of Ub-GFP quantification in Purkinje cell axons using ImageJ software. Statistical significance between loss of PI31 mice and control mice is p=0.0037 (two-tailed T test).

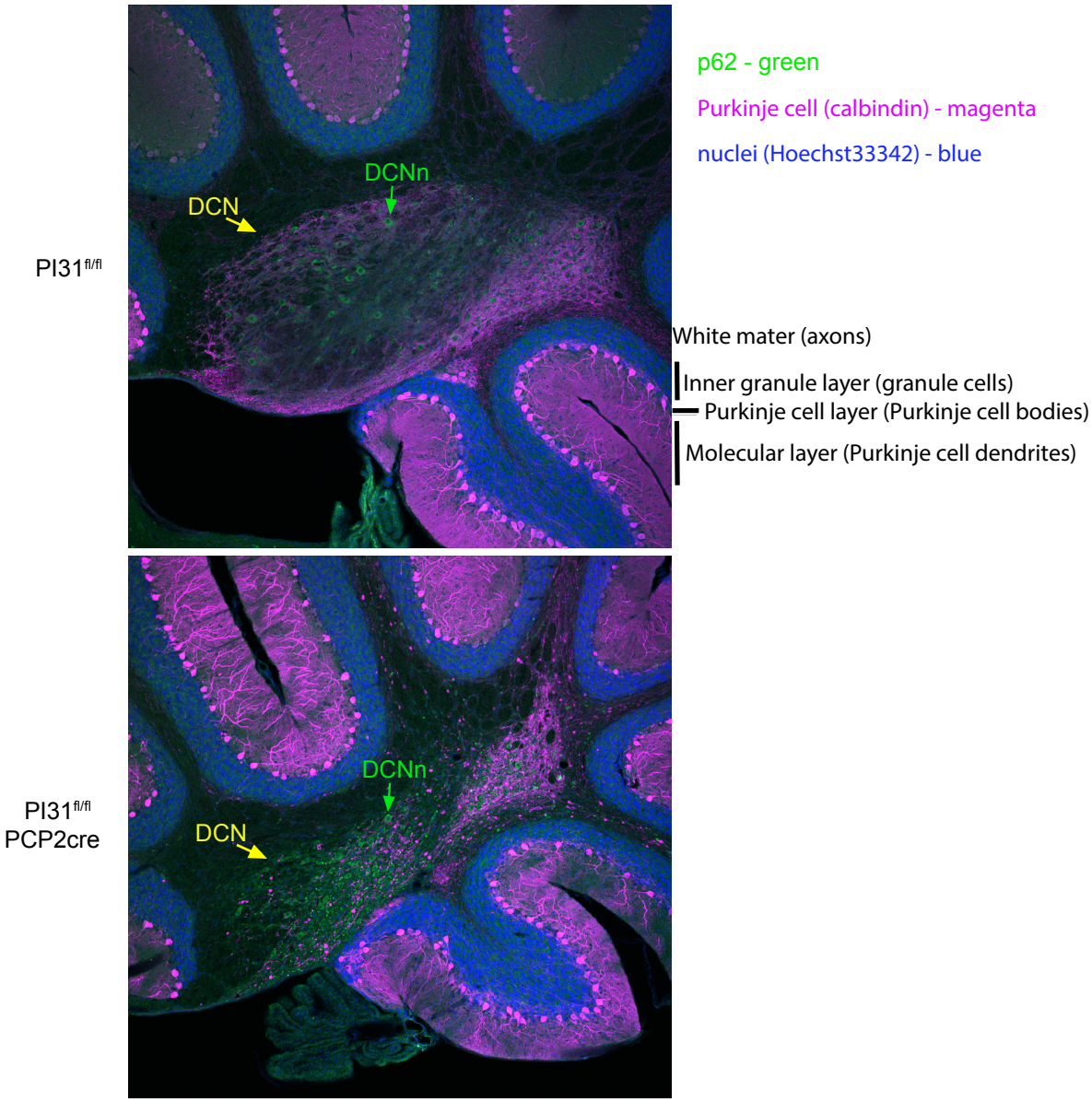

Fig. S7: *PI31$^{fl/fl}$ Pcp2-Cre* mice accumulate p62-positive aggregates only in the DCN.
Overview of p62-positive aggregates (green) which accumulated in the DCN (yellow arrow) of 2-month old *PI31$^{fl/fl}$ Pcp2-Cre* mice. The size of the DCN was reduced in *PI31$^{fl/fl}$ Pcp2-Cre* mice, presumably due to loss of PC axon terminals (magenta).

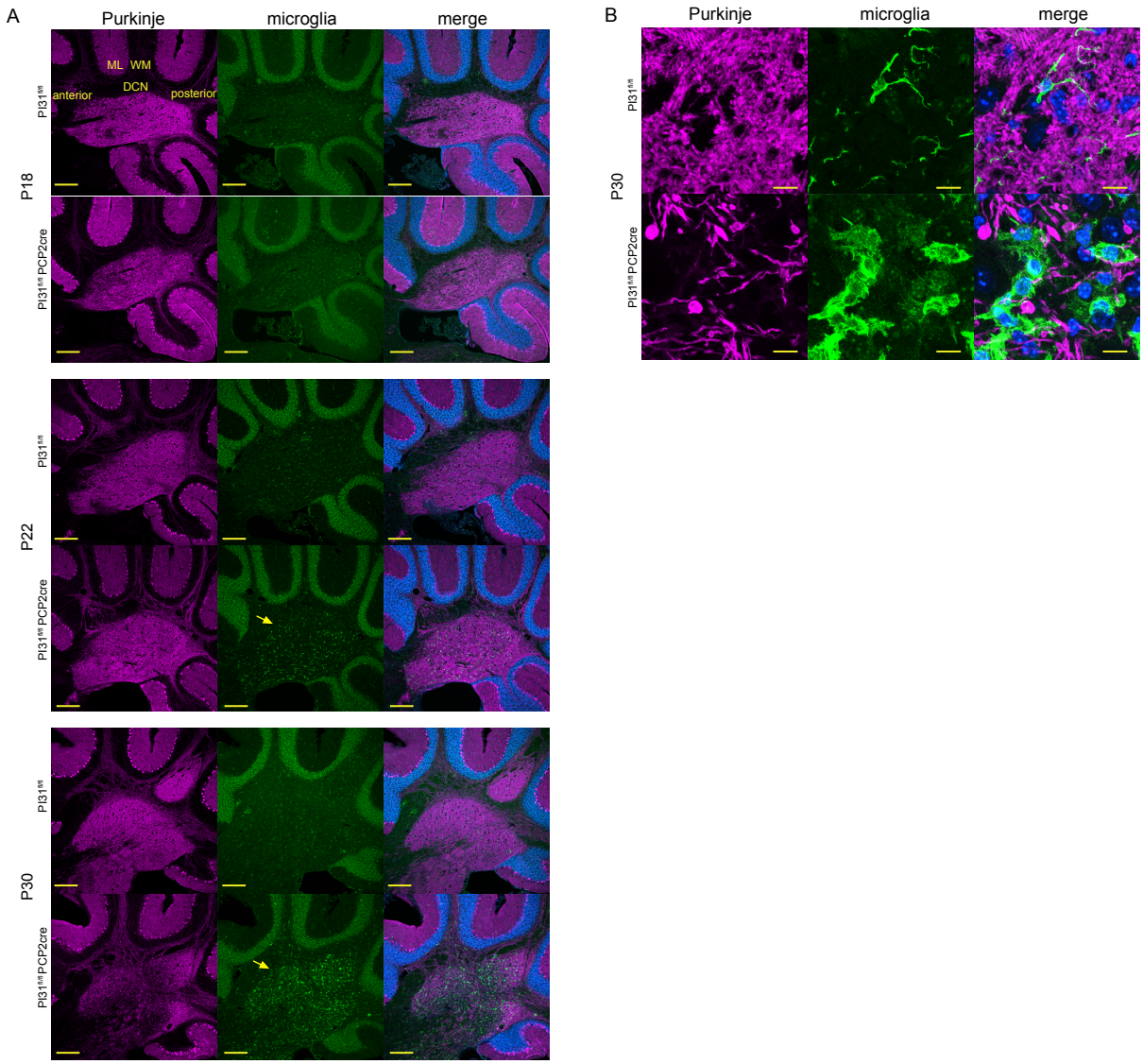

Fig. S8: Loss of PI31 in Purkinje cells leads to gliosis in the DCN of the cerebellum. (*A,B*) Cerebellum from P18, P22 and P30 *PI31*$^{fl/fl}$ and *PI31*$^{fl/fl}$ *Pcp2-Cre* mice stained with Calbindin (magenta) for Purkinje cells, Iba1 (green) for microglia, and Hoechst 33342 for nuclei (blue). (*A*) At P18, no difference is seen in microglia between *PI31*$^{fl/fl}$ *Pcp2-Cre* mice and their control littermates. However, at P22 we began to detect reactive microglia in the DCN of *PI31*$^{fl/fl}$ *Pcp2-Cre* mice. The number of reactive microglia greatly increased in the DCN of P30 *PI31*$^{fl/fl}$ *Pcp2-Cre* mice indicating the induction of gliosis. Yellow arrows point to reactive microglia in the DCN. Scale bar 100 µm. (*B*) Higher magnification of P30 mice DCN shows the morphologically distinct ramified microglia in *PI31*$^{fl/fl}$ control siblings and reactive microglia in *PI31*$^{fl/fl}$ *Pcp2-Cre* mice. DCN (deep cerebellar nuclei), ML (molecular layer) and WM (white matter). Scale bar 10 µm.

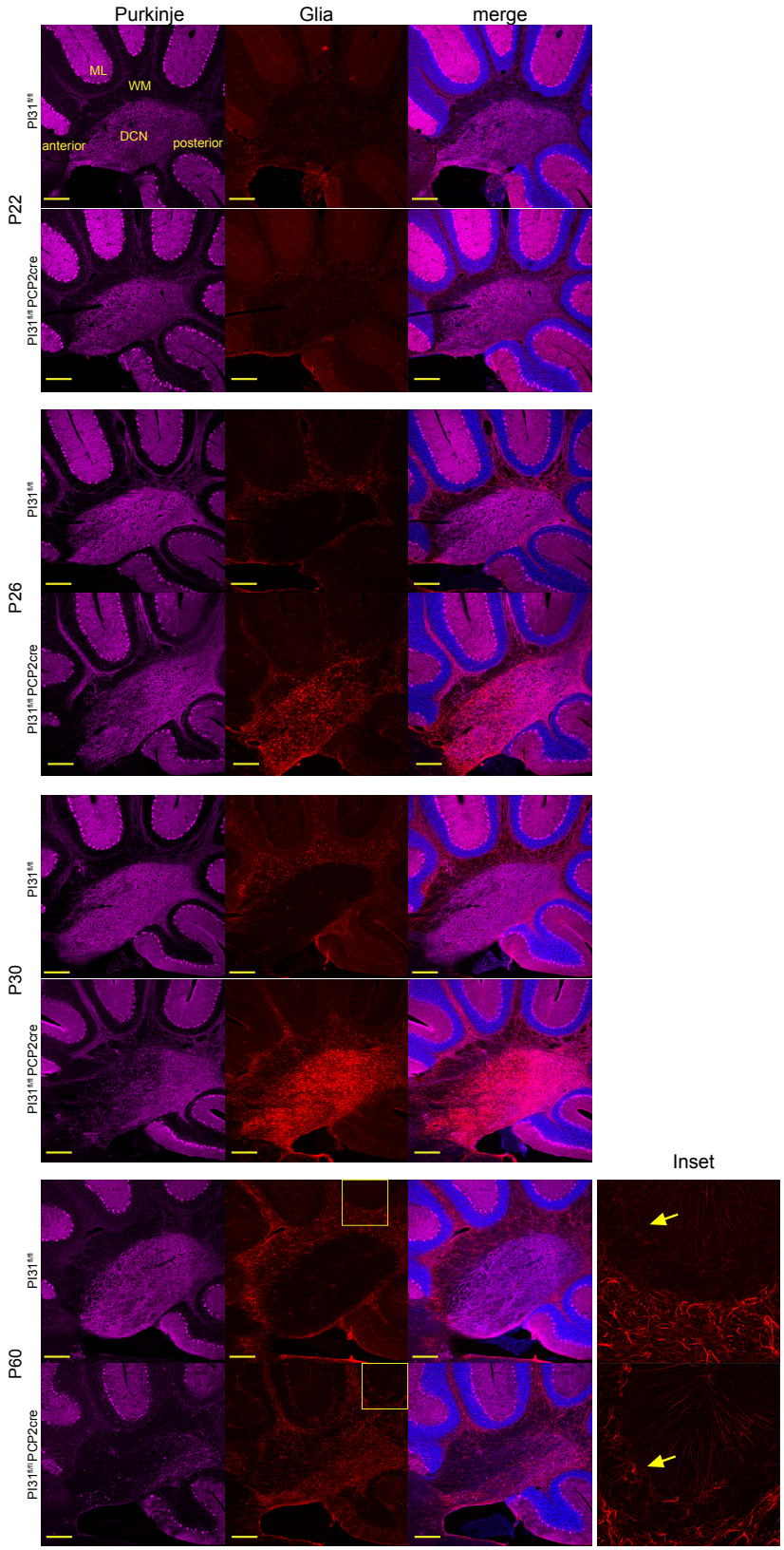
Purkinje
Glia
merge
Inset
P22
P26
P30
P60
PI31fl/fl
PI31fl/fl PCP2cre
ML
WM
DCN
anterior
posterior

Fig. S9: Loss of PI31 in Purkinje cells leads to astrogliosis in the DCN of the cerebellum. Cerebellum from P22, P26, P30 and P60 *PI31$^{fl/fl}$* and *PI31$^{fl/fl}$ Pcp2-Cre* mice stained with Calbindin (magenta) for Purkinje cells, GFAP (red) for glia, and Hoechst 33342 for nuclei (blue). PC axon terminals in the DCN of *PI31$^{fl/fl}$ Pcp2-Cre* looked normal at P22, but evidence of axon swelling and loss became apparent at P26 and prominent at P30. At P60, loss of axon terminals in *PI31$^{fl/fl}$ Pcp2-Cre* mice was nearly complete. GFAP staining in cerebellum of *PI31$^{fl/fl}$ Pcp2-Cre* mice was similar to that of control littermates at P22. At P26, gliosis in the DCN became apparent, and at P30 there was a dramatic increase of glia staining strongly for GFAP in the DCN. This effect appears to be specific to the PC axons, as it was not observed in the Purkinje or molecular layers. At P60, there were also some reactive astrocytes in the Purkinje layer (inset), coinciding with the onset of PC loss. DCN (deep cerebellar nuclei), ML (molecular layer) and WM (white matter). Scale bar 100 μm.

Antigen	Company	Cat#	Host	Isotype	WB	IHC-P/IF	HIER	Wholemount
Proteasome 20S alpha7 subunit (MCP72)	Enzo	BML-PW8110	Mouse	IgG1	1:5000			
Ubi-FK2	Enzo	BML-PW8810	Mouse	IgG1	1:1000	1:100-200		
P62	Abcam	ab56416	Mouse	IgG2a	1:2500	1:500	Sodium-Citrate pH 6.00	1:500 (Alexa647 Conjugated)
PI31	Abcam	ab140497	Rabbit		1:1000			
PI31	Thermo Fisher Sci.	PA5-18133	Goat		1:2000			
Actin (HRP conjugated)	Cell Signaling Tech.	5125S	Rabbit		1:10000			
Tuj1	BD Pharmingen	560339	Mouse	IgG2a				1:200 (Alexa488 Conjugated)
Synapsin	Cell Signaling Tech.	13197	Rabbit	IgG				1:200 (Alexa488 Conjugated)
Alpha-Bungarotoxin	Invitrogen	B-13423	N/A	N/A				100ng/ml
td-Tomato	SICGEN	AB8181-200	Goat			1:200	Sodium-Citrate pH 6.00	
FUS	Sigma-Aldrich	HPA008784	Rabbit	IgG		1:2000	Sodium-Citrate pH 6.00	
TDP43	Abcam	ab109535	Rabbit	IgG		1:1000	Sodium-Citrate pH 6.00	
Proteasome 19S Rpt3/S6b subunit (TBP7-27)	Enzo	BML-PW8765	Mosue	IgG1	1:1000			
Proteasome 19S Rpt1/S7 subunit (MSS1-104)	Enzo	BML-PW8825	Mouse	IgG1	1:3000			
Proteasome 20S alpha4 subunit (MCP79)	Enzo	BML-PW9140	Mouse	IgG1	1:2000			
Proteasome 20S alpha2 subunit (MCP21)	Enzo	BML-PW8105	Mouse	IgG1	1:2000			
Ub K48	Abcam	ab140601	Rabbit	IgG	1:1000			
GFP	Thermo Fisher Sci.	A11122	Rabbit	IgG		1:2000		
Calbindin	Swant	300	Mouse	IgG1		1:1000		
Calbindin	Hatten Lab		Rabbit	IgG		1:1000		
Calbindin	EnCor	CPCA-Calb	Chicken	IgY		1:1000		
NeuN	Abcam	ab207282	Rabbit	IgG		1:100		
GFAP	Dako	Z0334	Rabbit	IgG		1:500		
GFAP	EnCor	CPCA-GFAP	Chicken	IgY		1:2000		
Iba	Wako	019-19741	rabbit	IgG		1:1000		

Table S1 Antibody details

#	Mouse strain	Catolog#	Company	Notes
1	PI31CRISPR	N/A	Generated By This Lab	Available on request
2	PI31 KOF	Psmf1$^{tm1a(EUCOMM)Hmgu}$	EUCOMM	Generated from ES cells
3	PI31 floxed	Psmf1$^{tm1c(EUCOMM)Hmgu}$	EUCOMM	By Cross to FLP1 recombinase
4	FLP-1 Recombinase	5703	Jackson Laboratory	
5	CDX2-Cre	9350	Jackson Laboratory	
6	Hb9-Cre	6600	Jackson Laboratory	
7	UbcCre-ERT2	7001	Jackson Laboratory	
8	Pcp2-Cre	Tg(Pcp2-Cre)GN135Gsat	GENSAT (Rockefeller University)	
9	Ub-G76V-GFP	008111	Jackson Laboratory	
10	Ai9	7909	Jackson Laboratory	

Table S2. Mouse strains.

Strain	Forward	Reverse	Annealing Temp	Product Size
PI31 CRISPR	TGG CTT TGG CAC GGT CA	GGA AGT GGT GAC AAA CGG C	55	WT-111bp/CRISPR - 95bp
LacZ	ATT CCA GCT GAG CGC CGG TCG C	GCG AGC TCA GAC CAT AAC TTC GTA TA	55	400
Cre	CAG GGT GTT ATA AGC AAT CCC	CCT GGA AAA TGC TTC TGT CCG	55	250
PI31 floxed and WT	CAC CCT GGA CTG TGA ACA CC	TGT TTG CAC CTA AGA ACC CAG T	60	WT-250bp/ Floxed 400bp
GFP	GCA CCA TCT TCT TCA AGG ACG AC	TCT TTG CTC AGG GCG GAC TG	60	343
Ai9 (tdTomato)	CTG TTC CTG TAC GGC ATG G	GGC ATT AAA GCA GCG TAT CC	60	196
Rosa	AAG GGA GCT GCA GTG GAG TA	CCG AAA ATC TGT GGG AAG TC	60	297
FLPe	TGC CGG TCC TAT TTA CTC GT	TAC TTC TTT AGC GCA AGG GGT AG	60	100

Table S3 Genotyping primers

Step #	Temp °C	Time	Note
1	94	2 min	
2	94	20 sec	
3	65	15 sec	-0.5 C per cycle decrease
4	68	1 min	
5			repeat steps 2-4 for 10 cycles (Touchdown)
6	94	15 sec	
7	55	15 sec	
8	72	1 min	
9			repeat steps 6-8 for 28 cycles
10	72	2 min	
11	4		Hold

Table S4 Genotyping PCR program. This program was used with all primer sets.

Reagent	Company	Cat#
Purified Bovine 20S Proteasome	UBPBio	A1401
Purified Bovine 26S Proteasome	UBPBio	A1201
cOmplete, EDTA-free Protease Inhibitor Cocktail	Sigma-Aldrich	1E+10
PhosSTOP™- phosphatase inhibitor tablets	Sigma-Aldrich	5E+09
Native Sample Buffer	BIO-RAD	161-0738
3–8% Criterion™ XT Tris-Acetate Protein Gel, 26 well, 15 µl	BIO-RAD	345-0130
Immobilon-P Membrane, PVDF	Sigma-Aldrich	IPVH85R
Bortezomib	LC laboratories	B-1408
MG-132, Ready Made Solution	Sigma-Aldrich	M7449-1ML
PrestoBlue Cell Viability Reagent	Thermo Fisher Scientific	A13262
CAS-Block	Thermo Fisher Scientific	00-8120
Richard-Allan Scientific Neg-50™ Frozen Section Medium	Thermo Fisher Scientific	6502
Normal Donkey Serum	Jackson ImmunoResearch	017-000-121
Triton X-100	BIO-RAD	161-0407
ProLong® Diamond anti-fade mounting medium	Thermo Fisher Scientific	P36965
Vectashield	Vector Laboratories	H-1000

Table S5 Reagents

Movie S1 (separate file). Illustrating neuro-motor phenotype of Pl31$^{fl/fl}$ Cdx2-cre mice
14 days old *Pl31$^{fl/fl}$* and *Pl31$^{fl/fl}$ CDX2-cre* littermates. *Pl31$^{fl/fl}$ CDX2-cre* mice had sever neuromotor phenotypes, characterized by spasticity, rigid muscle tone, strong tremor and a severely impaired righting response.

Movie S2 (separate file). Illustrating motor defects of Pl31$^{fl/fl}$ Hb9-cre mice
5 month-old *Pl31$^{fl/fl}$* and *Pl31$^{fl/fl}$ Hb9-cre* littermates. *Pl31$^{fl/fl}$ Hb9-cre* mouse had kyphosis and breathing difficulties.

Movie S3 (separate file). Illustrating cerebellar behavioral defects in *Pl31$^{fl/fl}$ Pcp2-cre* mice
Movie of 7 month-old male *Pl31$^{fl/fl}$* and *Pl31$^{fl/fl}$ Pcp2-cre* siblings. The *Pl31$^{fl/fl}$ Pcp2-cre* mouse had a halting gait and lost balance frequently, falling back or to the left or right. *Pl31$^{fl/fl}$ Pcp2-cre* mouse has difficulty regaining balance as it falls to the left.