

Essential role of the unfolded protein response regulator GRP78/BiP in protection from neuronal apoptosis

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Neurodegenerative diseases are often associated with dysfunction in protein quality control. The endoplasmic reticulum (ER), a key site for protein synthesis, senses stressful conditions by activating the unfolded protein response (UPR). In this study we report the creation of a novel mouse model in which GRP78/BiP, a major ER chaperone and master regulator of UPR, is specifically eliminated in Purkinje cells (PCs). GRP78-depleted PCs activate UPR including the induction of GRP94, PDI, CHOP and GADD34, feedback suppression of eIF2 α phosphorylation and apoptotic cell death. In contrast to current models of protein misfolding in which an abnormal accumulation of ubiquitinated protein is prominent, cytosolic ubiquitin staining is dramatically reduced in GRP78-null PCs. Ultrastructural evaluation reveals that the ER shows prominent dilatation with focal accumulation of electron-dense material within the ER. The mice show retarded growth and severe motor coordination defect by week 5 and cerebellar atrophy by week 13. Our studies uncover a novel link between GRP78 depletion and reduction in cytosolic ubiquitination and establish a novel mouse model of accelerated cerebellar degeneration with basic and clinical applications. *Cell Death and Differentiation* (2010) 17, 488–498; doi:10.1038/cdd.2009.144; published online 9 October 2009

Neurodegenerative diseases are often associated with the formation of toxic intracellular protein aggregates; therefore, it is crucial to understand the factors that regulate their formation and degradation.¹ The unfolded protein response (UPR) is an evolutionarily conserved mechanism to allow cells to adapt to stress targeted to the endoplasmic reticulum (ER).² Thus, when the protein load exceeds the protein-folding capacity of the ER, UPR signaling pathways are induced. ER protein quality control is critical for cerebellar Purkinje cell (PC) survival^{3–5} and is linked to the Marinesco–Sjögren syndrome in humans.^{6,7} ER chaperone, GRP78, also referred to as BiP/HSPA5, is a central regulator of ER function and UPR.^{2,8,9} Tissue culture studies revealed that on ER stress, GRP78/BiP is released from ER transmembrane sensors (PERK, ATF6 and IRE1) resulting in the triggering of the UPR.¹⁰ However, the direct role of GRP78 in regulating the UPR *in vivo* and in neurodegeneration is unknown, in part because of the lethality of *Grp78* $-/-$ embryos.¹¹ PCs are the principal neurons and the sole output of the computational circuitry of the cerebellar cortex, providing signals required for balance, motor coordination and cognitive learning.¹² The recent discovery that mutations in *SIL1*, which encodes a nucleotide exchange factor for GRP78, cause progressive multisystem disorder, including cerebellar ataxia in humans^{6,7} and protein accumulation and PC death in *SIL1*-deficient

woozy mutant mice,³ provides the first hint that ER protein quality control in terminally differentiated neurons is critical for their survival. Interestingly, in wozzy mutant mice, GRP78 is upregulated in PCs,³ and other co-chaperones such as GRP170 (also known as ORP150) and P58^{IPK} (also known as DNAJc3) may serve as alternative nucleotide exchange factors for GRP78.^{13–16} To test directly the role of GRP78 in the UPR *in vivo* and in neurodegeneration, as well as the mechanism for its putative protective function, here we created a PC-specific knockout mouse model of GRP78. Our studies reveal that complete elimination of GRP78 in PCs leads to specific modulation of the UPR and reduction in cytosolic ubiquitination. GRP78 conditional knockout mice developed PC degeneration by 4.5 weeks, associating with severe motor coordination defect by week 5 and cerebellar atrophy by week 13, thereby establishing the requirement of GRP78 for PC survival and normal cerebellar function.

Results

Accelerated PC degeneration and cerebellar atrophy in *Grp78* conditional knockout mice. The *Grp78* PC-specific knockout mice (abbreviated below as *F*⁻; pc-Cre) were generated by crossing *Grp78* *F*⁻ mice¹⁷ with L7Cre-2

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Abbreviations: ER, endoplasmic reticulum; ERAD, ER-associated protein degradation; PCs, Purkinje cells; UPR, unfolded protein response

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transgenic mice.¹⁸ In the *Grp78* (-) allele, exons 5, 6 and 7 encoding the ATPase domain and peptide-binding domain essential for the chaperone function of GRP78 are deleted (Figure 1a), and no truncated protein was detected.¹¹ The genotypes of mice harboring the various *Grp78* alleles and the pc-Cre allele were determined by PCR (Figure 1b), and

cohorts of mice with genotypes (1) *Grp78* +/+, (2) *Grp78* F/F, (3) *Grp78* F/F; pc-Cre, (4) *Grp78* F/- and (5) *Grp78* F/-; pc-Cre were monitored for growth and development.

In agreement with previous studies that *Grp78* heterozygosity does not affect mouse development and normal organ function,^{11,19} F/- mice are phenotypically normal (data

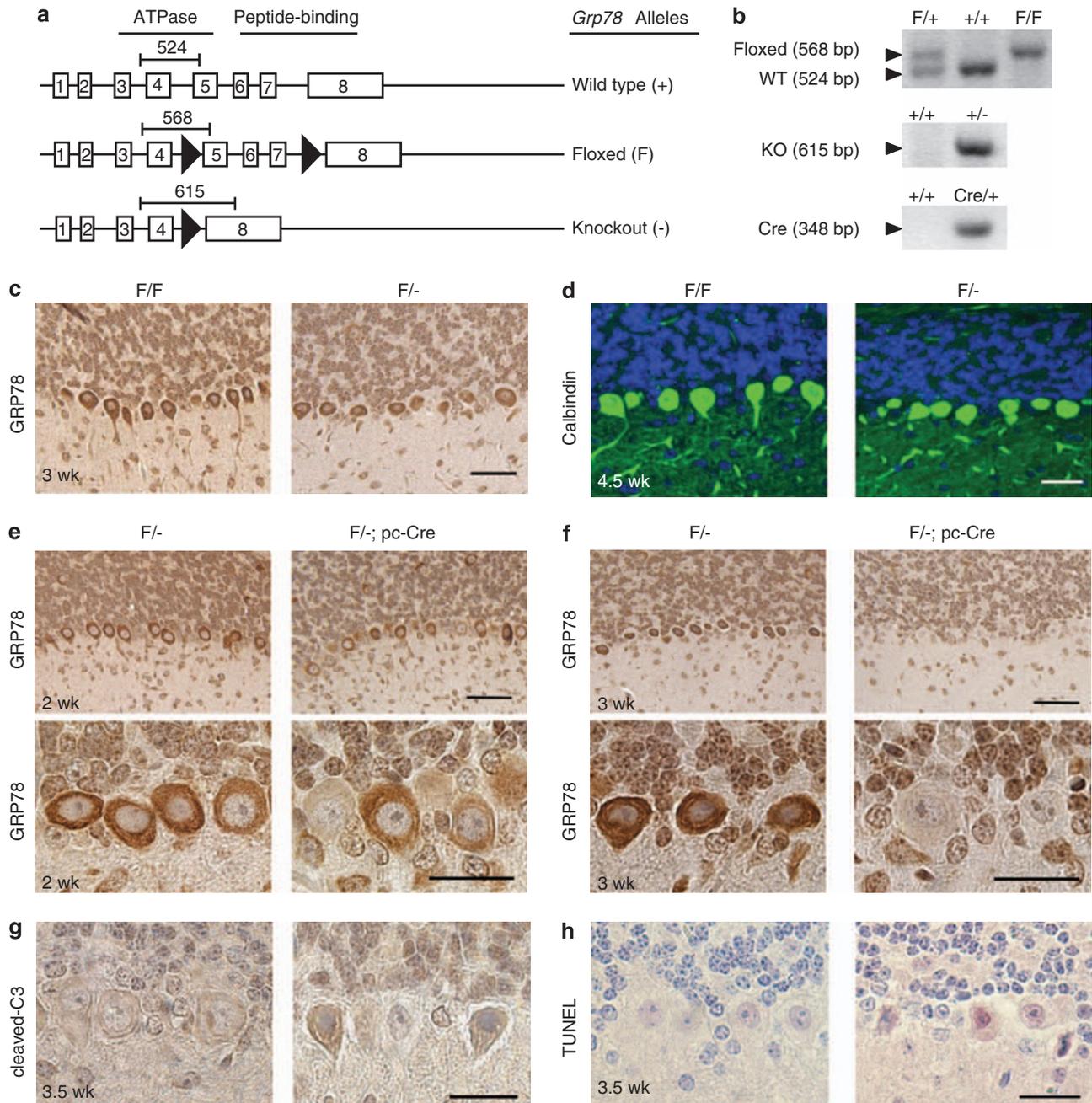


Figure 1 Generation of *Grp78* Purkinje cell (PC)-specific knockout mice. (a) Schematic drawing for the *Grp78* wild-type (wt) allele (+), floxed allele (F) and knockout (KO) allele (-). Black arrowheads indicate *loxP* sites. The bars with numbers indicate the size and position of PCR products. (b) Representative genotyping results of *Grp78* wt, floxed and KO alleles, and Cre transgene with expected PCR product size. (c) Representative immunohistochemical (IHC) staining for GRP78 shows similar expression levels of GRP78 in the PCs of *Grp78* F/F mice and their F/- littermates at 3 weeks of age. Staining was performed on sagittal sections of the cerebellum of *Grp78* F/F and F/- mice. Scale bar represents 50 μm. (d) Representative immunofluorescence (IF) staining for calbindin shows there is no PC degeneration in *Grp78* F/- mice compared with their F/F littermates at 4.5 weeks of age. Green: calbindin; blue: DAPI. Scale bar represents 50 μm. (e, f) Representative IHC staining for GRP78 on the cerebellum of *Grp78* F/- and their F/-; pc-Cre littermates at 2 weeks (e) and 3 weeks (f) of age. Scale bars represent 50 μm (upper panel) and 25 μm (lower panel). (g, h) Representative IHC staining for cleaved caspase-3 (g) and TUNEL (h) on PCs of *Grp78* F/- and their F/-; pc-Cre littermates at 3.5 weeks old. Scale bars represent 25 μm. For panels c-h, n = 3 mice per group were examined

not shown) and their PCs are intact and showed similar GRP78 staining as that of F/F mice (Figure 1c and d). In F/-; pc-Cre mice, Cre recombinase was expressed specifically in PCs after postnatal day 6, causing a deletion of exon 5 to exon 7 of *Grp78*. Immunohistochemical analysis revealed that at 2 weeks of age, GRP78 staining was partially reduced in about 10% of the PCs of F/-; pc-Cre mice compared with that of F/- control, and by week 3, GRP78 was undetectable in about 90% of PCs (Figure 1e and f). Caspase-3 activation and positive TUNEL staining were detected in about 10% of PCs at

week 3.5 (Figure 1g and h), suggesting the onset of PC apoptosis.

Staining for the PC-specific marker calbindin confirmed a partial loss of PCs at week 4.5 (Figure 2a), and by week 13, all 10 lobules of the cerebellum showed extensive PC degeneration (Figure 2b). Correlating with PC loss, cerebellar atrophy was evident in F/-; pc-Cre mice by week 13 (Figure 2c). Quantification of the age-dependent PC loss is summarized in Figure 2d. Collectively, these results establish that GRP78 is required for PC survival and cerebellum integrity.

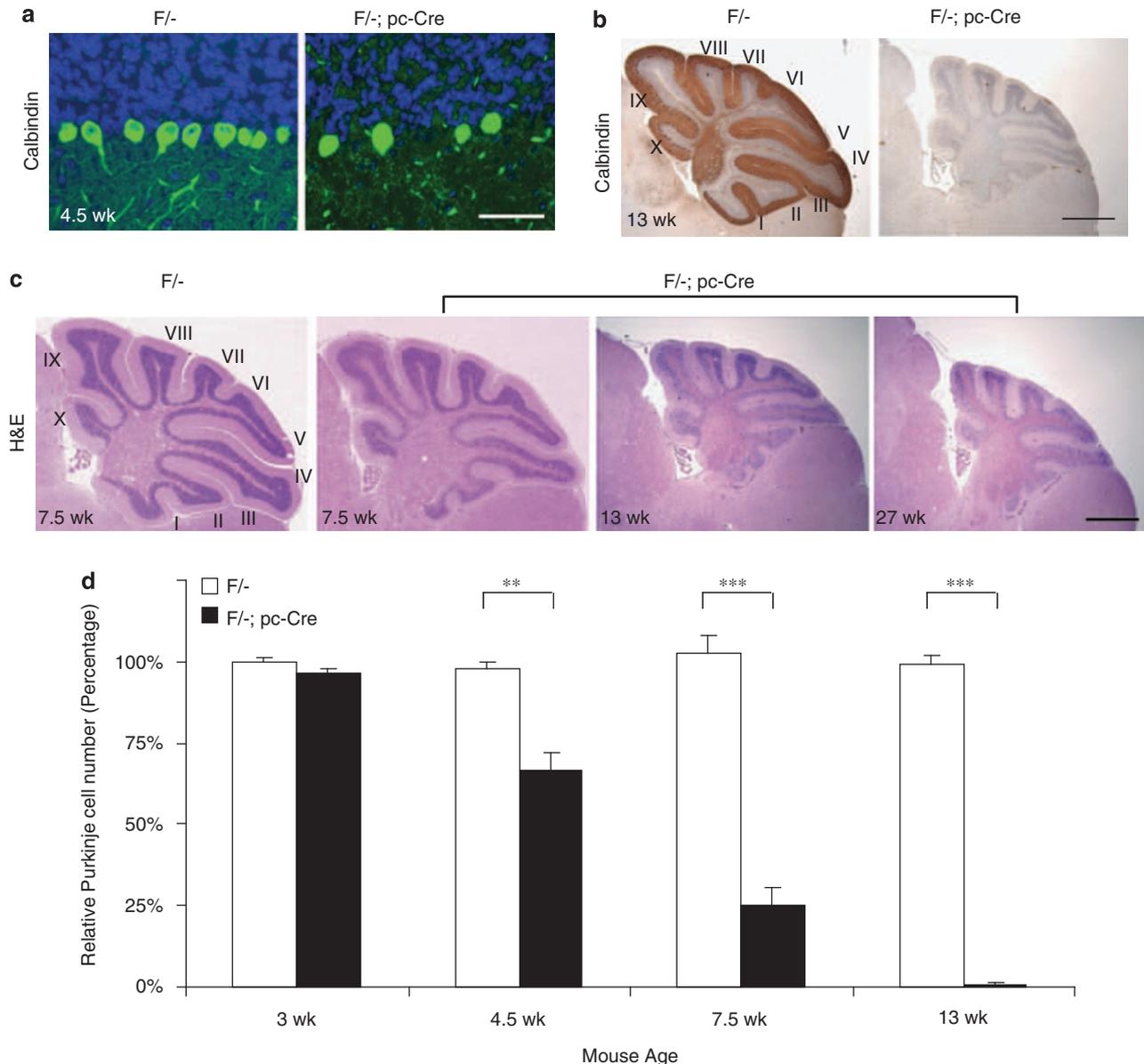


Figure 2 *Grp78* Purkinje cell (PC)-specific knockout mice show accelerated PC degeneration and cerebellar atrophy. (a) Representative immunofluorescence (IF) staining for calbindin shows PC degeneration in *Grp78* F/-; pc-Cre mice compared with their F/- littermates at 4.5 weeks of age. Green: calbindin, blue, DAPI. Scale bar represents 50 μ m. (b) Representative immunohistochemical (IHC) staining for calbindin on the cerebellum sections of *Grp78* F/- mice and their F/-; pc-Cre littermates at 13 weeks of age. Sections were counterstained with hematoxylin. Cerebellar lobules are indicated by Roman numerals. Scale bar represents 1.5 mm. (c) Hematoxylin and eosin (H&E) staining on the cerebellum sections of *Grp78* F/- mice and their F/-; pc-Cre littermates at indicated ages, demonstrating cerebellar atrophy. Scale bar represents 1.5 mm. (d) Age-dependent PC degeneration in *Grp78* F/-; pc-Cre mice compared with their F/- littermates. The number of PCs determined by H&E staining was quantitated within the same areas of cerebellar sections and plotted against the age of mice. The number of PCs in 3-week-old F/- mouse cerebellum was set as 100%. Data are presented as mean \pm S.E.M. ** $P \leq 0.01$, *** $P \leq 0.001$ (Student's *t*-test). For all panels, $n = 3$ mice per group were examined

Severe motor coordination defects in *Grp78* PC knockout mice. Although *F*^{-/-}; *pc-Cre* mice were born with the expected Mendelian ratio and survive as well as *F*/*F* or *F*^{-/-} siblings, both male and female mice developed postnatal growth retardation when compared with *F*^{-/-} mice (Supplementary Figure S1), such that there was a significant decrease in overall body size (Figure 3a) and fasting body weight (Figure 3b). Food consumption of *F*^{-/-}; *pc-Cre* mice was also less than that of *F*^{-/-} mice, which might in part account for the lower body weight (Figure 3c). By week 5.5, both male and female *F*^{-/-}; *pc-Cre* mice exhibited motor defects indicative of cerebellar ataxia. They had difficulty walking in a straight line; even while standing still

the mice could not maintain balance and seemed 'tipsy' (see Supplementary Information video). The rota rod test confirmed severely impaired motor coordination ability in both male and female cohorts of *F*^{-/-}; *pc-Cre* mice, consistently over multiple trials (Figure 3d). Footprint recording further revealed an ataxic walking pattern (Figure 3e), with reduced stride/width ratio compared with *F*^{-/-} mice (Figure 3f). Interestingly, none of these phenotypes were detected in mice with PC-specific knockout of GRP94, another major ER chaperone (Supplementary Figure S2). Thus, not only that GRP78 function cannot be compensated by GRP94 but also GRP78 is uniquely required for PC survival.

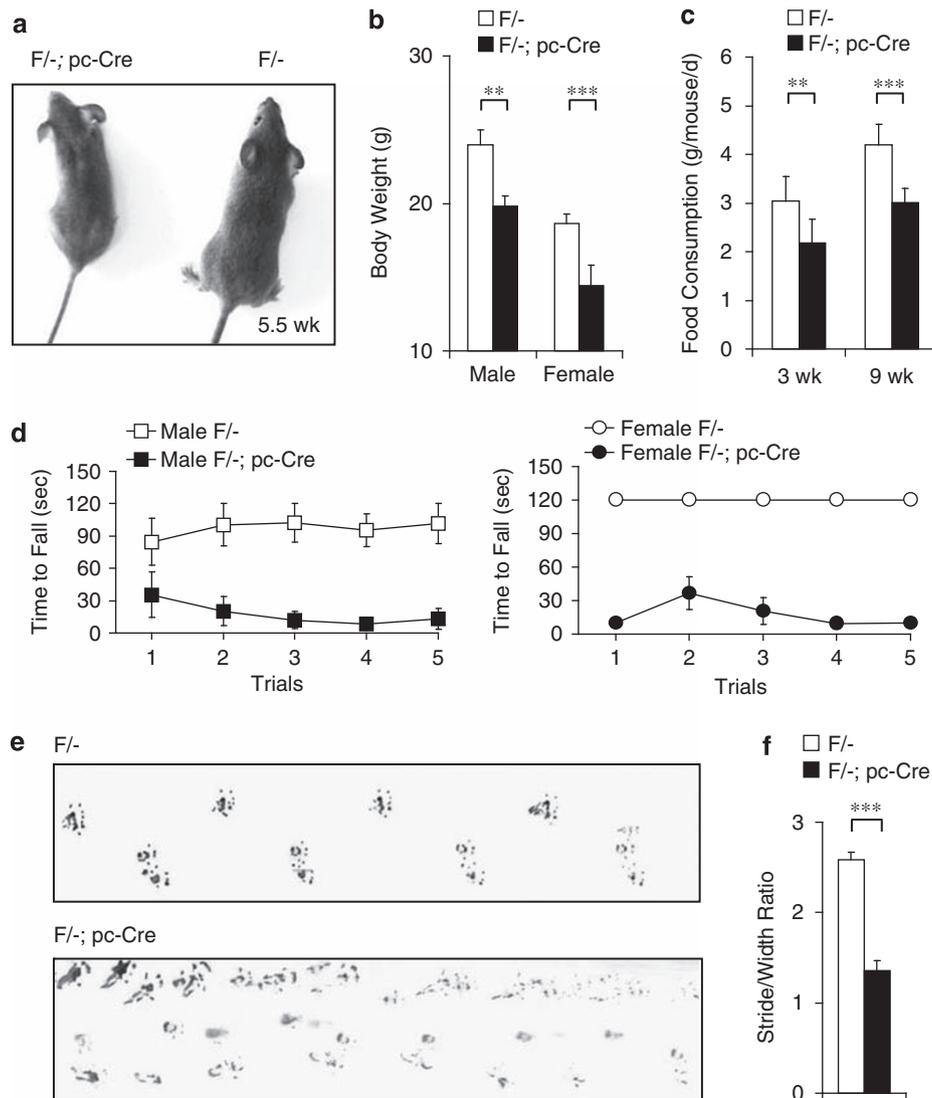
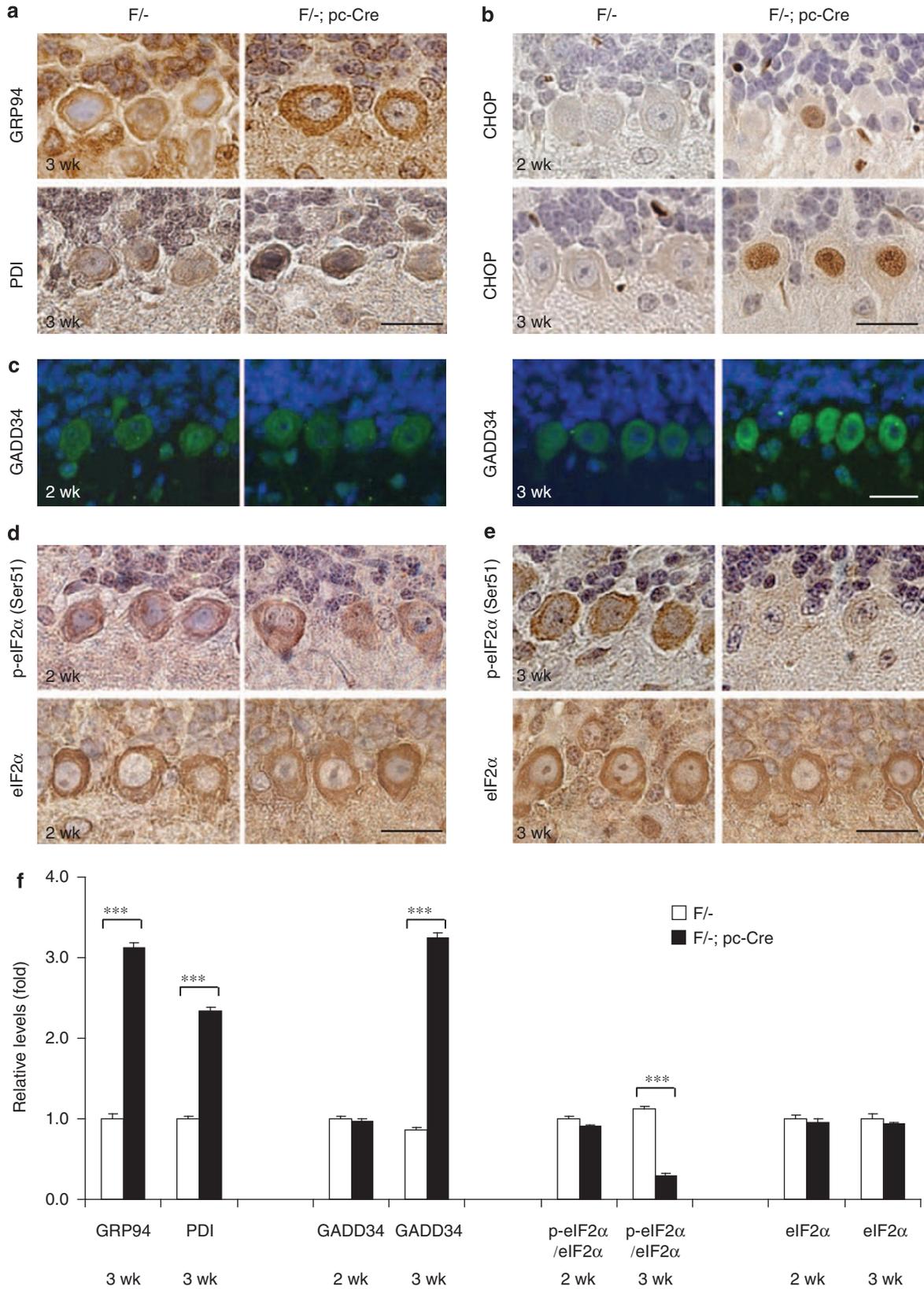


Figure 3 Behavioral abnormalities of *Grp78* conditional knockout mice. (a) A representative male *Grp78* *F*^{-/-}; *pc-Cre* mouse and its *F*^{-/-} littermate at 5.5 weeks of age. (b) Fasting body weight of 10-week-old *Grp78* *F*^{-/-} mice and their *F*^{-/-}; *pc-Cre* littermates, both male and female; *n* = 10 mice per group. (c) Food consumption of male *Grp78* *F*^{-/-} mice and their *F*^{-/-}; *pc-Cre* littermates at 3 and 9 weeks of age. The food intakes for each individual mouse were measured for 7 consecutive days. *n* = 4 mice per group. (d) Rota rod test of *Grp78* *F*^{-/-} mice (open symbols) and their *F*^{-/-}; *pc-Cre* littermates (closed symbols) at 5.5 weeks of age. The time to fall off the rod (rotating at 30 r.p.m.) at each trial is recorded; *n* = 4 to 5 mice per group. *P*-value = 0.016 (male mice) and *P*-value = 0.008 (female mice) (randomization test). (e) Representative footprint records of *Grp78* *F*^{-/-} male mice and their *F*^{-/-}; *pc-Cre* male littermates at 5.5 weeks of age. The forelimbs and hindlimbs were painted blue and red, respectively. (f) The ratios of stride lengths to paw-based widths from the footprint test; *n* = 4 mice per group. Data in panels b–d and f are all presented as mean ± S.E.M. ***P* ≤ 0.01, ****P* ≤ 0.001 (Student's *t*-test). A color version of this figure is available online



Modulation of UPR signaling pathways in GRP78-null PCs. The ability to genetically inactivate both *Grp78* alleles in PCs provides a unique experimental system to test directly how depletion of GRP78 affects the UPR in terminally differentiated neurons, in which ER protein quality control is central to their survival.^{20,21} One of the prosurvival branches of the UPR upregulates ER chaperones, such as GRP78 and GRP94, and folding enzymes, such as PDI, to alleviate protein misfolding.²² We observed that GRP94 and PDI were upregulated in almost all PCs of 3-week-old *F/-*; pc-Cre mice (Figure 4a). Immunostaining revealed that CHOP, an ER stress-inducible transcription factor,²³ was induced in about 5% of PCs in 2-week-old *F/-*; pc-Cre mice; however, by 3 weeks of age, about 90% of PCs were CHOP positive (Figure 4b). ER stress activates the PERK signaling pathway, which leads to transient global translational arrest through phosphorylation of the initiation factor eIF2 α ; however, this can be inhibited by a feedback mechanism mediated by GADD34, a downstream target of CHOP.²⁴ Corresponding to CHOP induction and GADD34 upregulation in the PCs of *F/-*; pc-Cre mice (Figure 4c), eIF2 α phosphorylation, which was observed at 2 weeks, was dramatically reduced by 3 weeks with the overall level of eIF2 α unaffected (Figure 4d and e). As summarized in Figure 4f, quantitation of GRP94, PDI, GADD34, phosphorylated eIF2 α and eIF2 α immunostaining indicates that GRP78 depletion significantly upregulates GRP94, PDI and GADD34 and suppresses eIF2 α phosphorylation without affecting the eIF2 α level in PCs of *F/-*; pc-Cre mice compared with their *F/-* littermates. In contrast, knockout of *Grp94* did not induce UPR signaling pathways in mouse embryonic stem cells (mESCs) (Supplementary Figure S3), correlating with no neurological phenotypes in *Grp94* PC-specific knockout mice. Under thapsigargin-induced ER stress, *Grp94*^{-/-} and ^{+/+} mESCs exhibited no difference in phosphorylation of PERK and eIF2 α , induction of GADD34 and splicing of XBP-1, further supporting that GRP94 did not affect UPR signaling (Supplementary Figure S3).

Reduction of cytosolic ubiquitin staining and ER expansion in GRP78-null PCs. In the mammalian ER-associated protein degradation (ERAD) mechanism, misfolded proteins released from calnexin are captured by a recognition complex containing EDEM, then passed on to GRP78 and PDI before unfolding and transport to the cytosol through the retrotranslocation mechanism, polyubiquitinated by the E1-E2-E3 system and degraded by the proteasome.^{25,26} For both *F/-* and *F/-*; pc-Cre mice, ubiquitin staining was detectable in both the nucleus and cytoplasm in 2-week-old cerebellar PCs (Figure 5a).

Strikingly, despite upregulation of GRP94, PDI, GADD34 and CHOP, and a readily detectable level of other cellular proteins including calbindin, eIF2 α and β -actin (Figures 2a, 4 and data not shown), cytosolic ubiquitin staining was nearly undetectable in the PCs of *F/-*; pc-Cre mice by 3 weeks (Figure 5a). Quantitation of the relative ubiquitin levels shows a significantly reduced level of ubiquitin in *F/-*; pc-Cre mice at 3 weeks of age compared with their *F/-* littermates (Figure 5b). The immunohistochemical staining results were confirmed by immunofluorescence analysis, which also showed that, although ubiquitin staining was observed in *F/-* PCs, its level was reduced in the PCs of *F/-*; pc-Cre mice by week 2.5 and was barely detectable by week 3 (Figure 5c). The quantitation of ubiquitin immunofluorescence indicates the gradual loss of ubiquitin level in the PCs of *F/-*; pc-Cre mice compared with their *F/-* littermates (Figure 5d). Reduction of ubiquitin staining was further confirmed in GRP78-null prostate epithelial cells in another *Grp78* conditional knockout mouse model (Supplementary Figure S4).¹⁷ As GRP78 knockout in these cells does not affect prostate function or gross morphology,¹⁷ reduction in ubiquitin staining as a result of GRP78 depletion is unlikely the consequence of cells undergoing the cell death process.

p62/SQSTM1 is a cytosolic protein that binds to polyubiquitinated proteins and targets them to the autophagy mechanism for degradation.²⁷ The polyubiquitinated protein aggregates stabilize p62.²⁸ Consistent with the reduction in the ubiquitinated protein level, p62 level was reduced in 3-week-old GRP78-null PCs, as compared with PCs from either 2- or 3-week-old *F/-* mice, or 2-week-old *F/-*; pc-Cre mice, in which GRP78 expression was still detected (Figure 5e). Quantitation of p62 immunostaining levels in *F/-* and *F/-*; pc-Cre mice showed a significant reduction by week 3 (Figure 5f). In contrast, PCs from *F/-* and *F/-*; pc-Cre mice showed similar levels of calnexin, which is an ER protein, and neurofilament and calbindin, which are cytoskeletal and cytosolic proteins, respectively (Figure 6).

Transmission electron microscopy (EM) of PCs from 3.5-week-old *F/-*; pc-Cre mice further revealed that the ER showed prominent dilatation with accumulation of flocculent material, and in some cases, focal accumulation of electron-dense material (Figure 7a and Supplementary Figure S5). No double membrane-bound inclusions were seen that would be suggestive of autophagy. ImmunoEM analysis using antibody against calnexin, an ER transmembrane protein, showed that in *F/-* mice, typical tubular ER structures were labeled; whereas in *F/-*; pc-Cre mice, expanded ER structures enclosing electron dense or flocculent materials were labeled (Figure 7b). This confirms the ER identity of the expanded

Figure 4 Modulation of UPR pathways in Purkinje cells (PCs) of *Grp78* conditional knockout mice. (a, b) Representative immunohistochemical (IHC) staining for GRP94 and PDI (a) and CHOP (b) on the cerebellum sections of *Grp78* *F/-* mice and their *F/-*; pc-Cre littermates at indicated ages. (c) Representative immunofluorescence (IF) staining for GADD34 on the cerebellum sections of *Grp78* *F/-* mice and their *F/-*; pc-Cre littermates at 2 and 3 weeks of age. Green: GADD34, blue: DAPI. (d, e) Representative IHC staining for p-eIF2 α (Ser51) and eIF2 α on the cerebellum sections of *Grp78* *F/-* mice and their *F/-*; pc-Cre littermates at 2 and 3 weeks of age. IHC and IF staining were counterstained with hematoxylin and DAPI, respectively. All scale bars represent 25 μ m. For panels a–e, *n* = 3 mice per group were examined. (f) Quantitation of IHC and IF stainings is shown in panels a–e. The relative protein levels were quantitated by NIH software ImageJ (Bethesda, MD, USA). The mean level of staining in each group was determined by 40 randomly selected areas within PCs on the IHC or IF stained sections. The level of p-eIF2 α was normalized to eIF2 α . For GRP94 and PDI, the staining of PCs in *F/-* mice at 3 weeks of age was set as 1. For GADD34, p-eIF2 α and eIF2 α , the staining of PCs in *F/-* mice at 2 weeks of age was set as 1. Data are presented as mean \pm S.E.M. ****P* \leq 0.001 (Student's *t*-test)

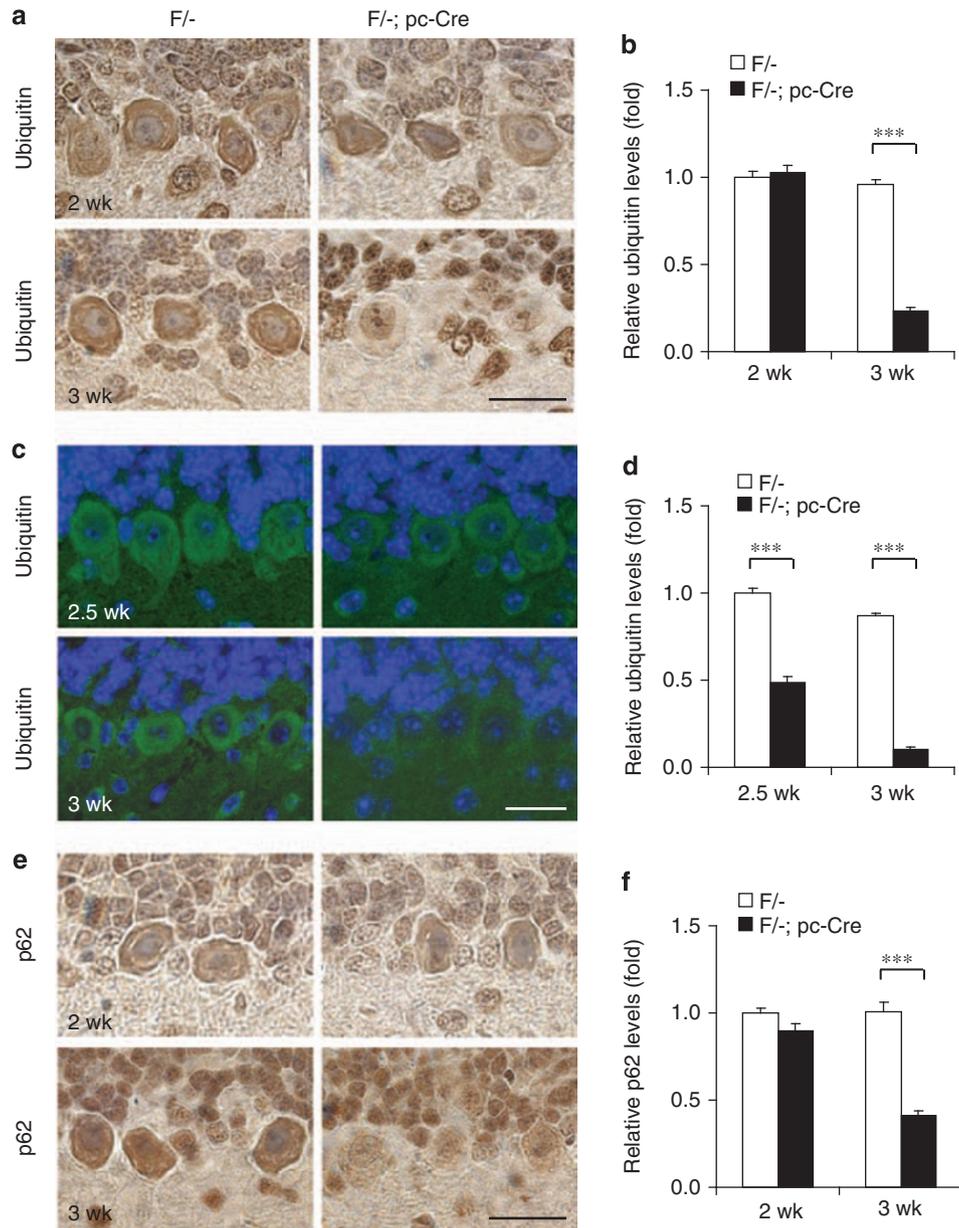


Figure 5 Reduction of protein ubiquitination and the p62 level in Purkinje cells (PCs) of *Grp78* conditional knockout mice. (a) Representative immunohistochemical (IHC) staining for ubiquitin on the cerebellum sections of *Grp78* F/- mice and their F/-; pc-Cre littermates at 2 and 3 weeks of age. (b) Quantitation of ubiquitin immunostaining, with the level of ubiquitin staining in the PCs of F/- mice at 2 weeks of age set as 1. (c) Representative immunofluorescence (IF) staining for ubiquitin on the cerebellum sections of *Grp78* F/- mice and their F/-; pc-Cre littermates at 2.5 and 3 weeks of age. (d) Quantitation of ubiquitin immunofluorescence, with the level of ubiquitin staining in the PCs of F/- mice at 2.5 weeks of age set as 1. (e) Representative IHC staining for p62 on the cerebellum sections of *Grp78* F/- mice and their F/-; pc-Cre littermates at 2 and 3 weeks of age. (f) Quantitation of p62 immunostaining with the level of p62 staining in the PCs of F/- mice at 2 weeks of age set as 1. All relative levels shown in panels b, d and f were quantitated by NIH software ImageJ. The mean level of staining in each group was determined by 40 randomly selected areas within PCs on IHC- or IF-stained sections. For all panels, $n = 3$ mice per group. Data in panels b, d and f are all presented as mean \pm S.E.M. *** $P \leq 0.001$ (Student's *t*-test). All scale bars represent 25 μ m

structures in PCs of F/-; pc-Cre mice. The alterations in UPR pathways and pathology observed in *Grp78* F/-; pc-Cre mice are summarized in Figure 8.

Discussion

The creation of the floxed *Grp78* mouse model provides a valuable tool whereby the function of GRP78, a major

molecular chaperone and key rheostat for ER homeostasis,²⁹ can be examined in the context of mammalian development and in specific adult tissues and organs. Previous studies showed that homozygous knockout of *Grp78* in mouse embryos results in proliferative defect and apoptosis of the inner cell mass, resulting in lethality at E3.5.¹¹ Nonetheless, specific knockout of *Grp78* in mouse prostate epithelial cells does not affect their viability,¹⁷ and in tissue culture cell lines,

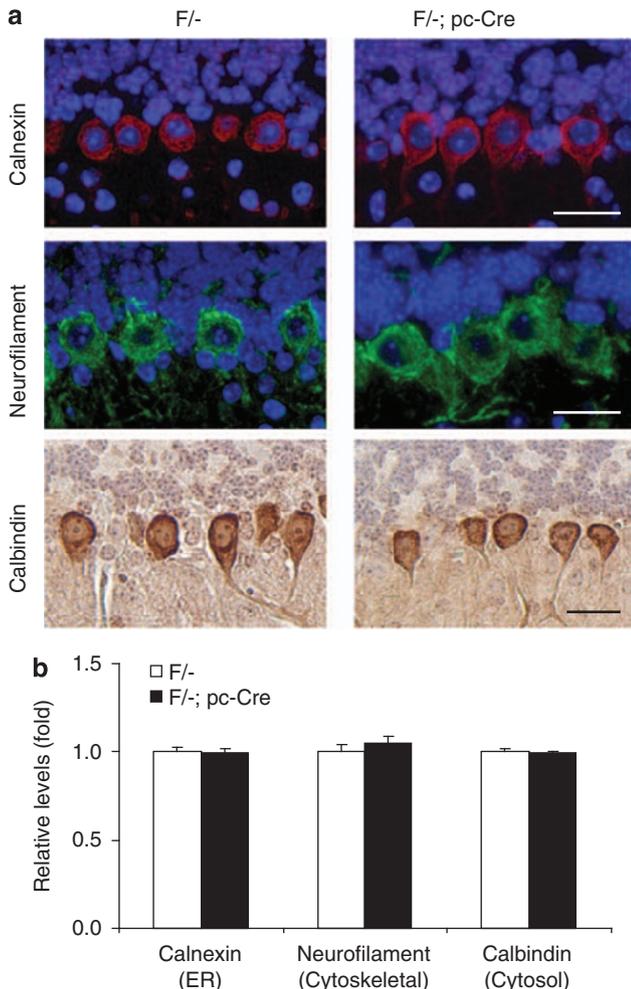


Figure 6 Similar expression levels of calnexin, nonphosphorylated neurofilament and calbindin in the Purkinje cells (PCs) of wild-type and mutant mice. **(a)** Representative immunofluorescence (IF) for calnexin and neurofilament and immunohistochemical (IHC) staining for calbindin on the cerebellum sections of *Grp78* F^{-/-} mice and their F^{-/-}; pc-Cre littermates at 3 weeks of age. All scale bars represent 25 μ m. **(b)** Quantitation of IF and IHC stainings shown in panel **a**. The relative protein levels were quantitated by NIH software ImageJ. The mean level of staining in each group ($n=3$ mice per group) was determined by 40 randomly selected areas within PCs on IF- or IHC-stained sections. For all quantitations, the staining of PCs in F^{-/-} mice at 3 weeks of age was set as 1. Data are presented as mean \pm S.E.M.

knockdown of GRP78 by siRNA only induces apoptosis in two out of seven human cell lines being tested.³⁰ Thus, the requirement of GRP78 in adult organs and tissues remains to be determined. Here, through the creation of the F^{-/-}; pc-Cre mouse, in which *Grp78* is specifically knocked out in terminally differentiated, nonproliferating PCs, and through examination of the resulting mouse phenotypes, several novel observations are made. As evidenced in phenotypically normal F^{-/-} mice, one functional allele of *Grp78* is sufficient for PC survival and cerebellum integrity, as these mice maintain normal motor function up to the age of 10 months. Interestingly, for mice over 10 months, we observed that, although female F^{-/-} mice maintain normal motor function, male F^{-/-} mice exhibit mild signs of motor deficiency on the basis of rotor rod tests (our unpublished results), suggesting an age-dependent

requirement for GRP78 in maintaining cerebellum function in heterozygous males, which warrants further confirmation and investigation.

On knockout of both alleles of *Grp78* in PCs, our studies revealed that mice show growth retardation, PC apoptosis, ataxia and severe defect in motor coordination by week 5 and cerebral atrophy by week 13, directly demonstrating that GRP78 is required for survival of PCs and cerebellum integrity. Nonetheless, there are distinct differences between *Grp78* F^{-/-}; pc-Cre mice (referred below as the 'tipsy' mice) and SIL1-deficient 'woozy' mice, which also suffered from ER stress. A likely explanation is that SIL1 is a nucleotide exchange factor for GRP78, but is not the only one. In the GRP78 conditional knockout mouse model, PC degeneration starts at week 4.5 and all 10 lobules degenerate simultaneously and completely by 3 months, revealing that GRP78 function cannot be substituted by any other factor in all 10 lobules. In contrast, onset of PC degeneration in wozzy mice starts at 3 months, and PCs in lobule X and caudal lobule IX are intact even at 1 year of age and older.³ The differences in PC death kinetics and the surviving lobule pattern suggest that there are other factors that maintain GRP78 function in the PCs of wozzy mice. Consistent with earlier reports that GRP170/ORP150 can serve as a nucleotide exchange factor for GRP78,^{13,14} it was recently discovered that brain expression of the GRP170 transgene is able to fully suppress the PC death phenotype caused by an SIL1 mutation (S Ackerman, personal communication). Furthermore, the PCs of wozzy mice have highly elevated levels of GRP78 compared with wild-type control because of ER stress. Thus, despite SIL1 disruption, the residual GRP78 function in the PCs of wozzy mice could mitigate the pathology and rapid loss of cerebellum function because of a complete lack of GRP78 in tipsy mice.

A previous demonstration that GRP78 depletion results in ER stress signaling was derived from ER stress titration or siRNA studies in tissue culture cells.^{10,30,31} The ability to completely eliminate GRP78 in PCs through genetic knockout in tipsy mice offers a unique opportunity to interrogate how a nonproliferating, terminally differentiated neuron responds to the complete loss of this UPR regulator. Although it is not possible to perform biochemical analysis as PCs constitute only a single-cell layer in the cerebellum, the large size of the PC with distinct nuclear and cytosolic compartments enabled us to use immunostaining techniques to determine the status of UPR that kinetically corresponds to the gradual depletion of GRP78 from these cells after activation of the cre-recombinase about 1 week after birth. We noted that it took about 2 weeks for GRP78 to be completely depleted from PCs. As a compensatory measure to GRP78 deficiency, ER chaperone GRP94 and folding enzyme PDI are upregulated, consistent with previous reports in *Grp78* heterozygous mouse embryo fibroblasts¹¹ and in established cell lines in which GRP78 was knocked down by siRNA.^{30,31} Also, as expected, CHOP is induced, confirming that GRP78 is a dominant suppressor of CHOP induction.³² Although the eIF2 α level was not changed in PCs of tipsy mice, eIF2 α phosphorylation was substantially reduced in GRP78-null PCs. One contributing factor could be the upregulation of GADD34, which is a downstream target of CHOP and a dominant inhibitor of eIF2 α phosphorylation. Unfortunately, using commercially available antibodies, we

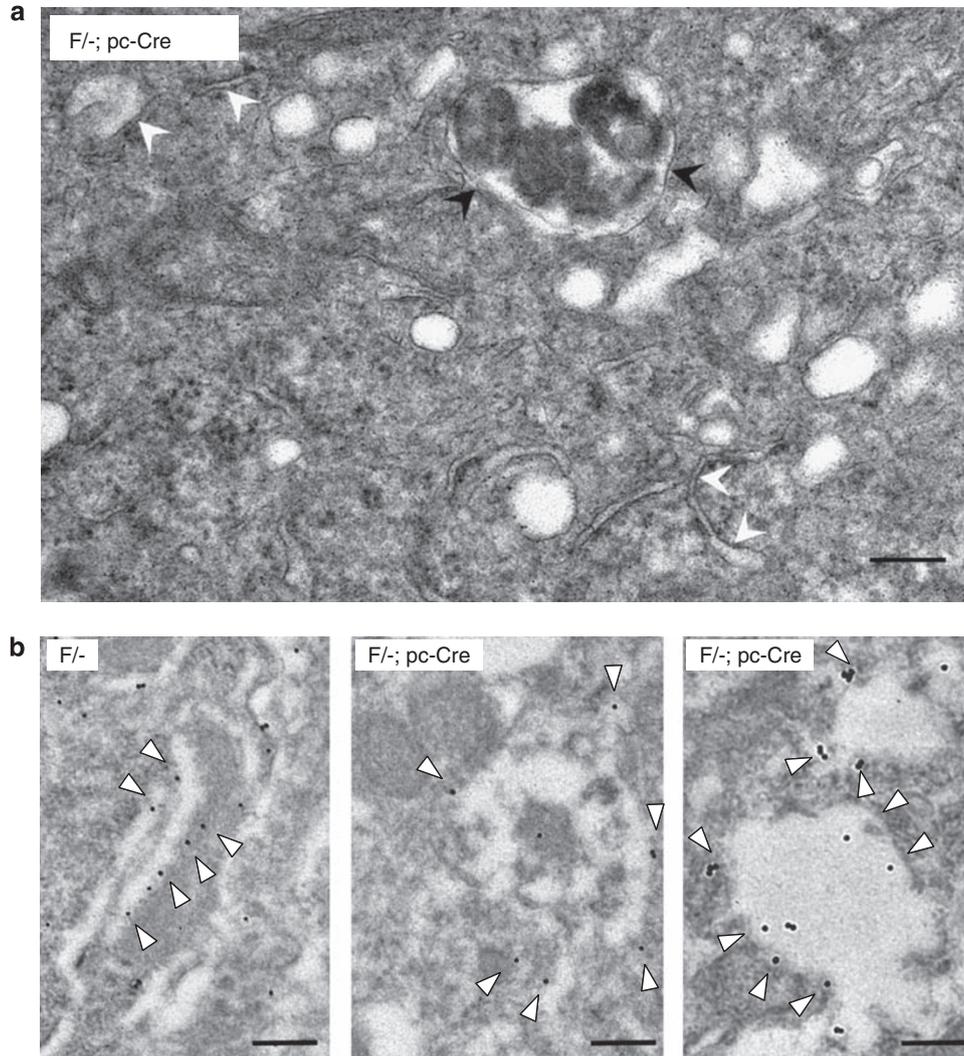


Figure 7 Altered ER structure in GRP78-null Purkinje cells (PCs). (a) Representative area of electron micrograph of a 3.5-week-old *F^{-/-}; pc-Cre* mouse cerebellum shows ER expansion and electron dense aggregates inside the ER in PCs. White arrowheads indicate a typical ER structure undergoing expansion. Grossly expanded ER contains diffuse accumulation of flocculent material, and in some cases, electron dense aggregates are prominent inside the ER (indicated by black arrowheads). Scale bar represents 0.5 μm . (b) Examples of electron micrographs showing immunogold labeling. PCs from 4.5-week-old *F^{-/-}* mice and their *F^{-/-}; pc-Cre* littermates were labeled for the ER marker calnexin. The 15 nm immunogold particles are indicated by open arrows. All scale bars represent 0.2 μm

were unable to detect in tissue sections the phosphorylation level of PERK, which is an upstream regulator of eIF2 α phosphorylation (data not shown). Although further studies are required to determine whether alterations in the chaperone balance and UPR markers in GRP78-null PCs contribute to the pathology of *Grp78 F^{-/-}; pc-Cre* mice, in this study we showed that knockout of *Grp94* has no effect on ER stress signaling, correlating with no PC loss and no neurological phenotype.

An unusual observation in the PCs of tipsy mice is that the level of cytosolic ubiquitin staining is dramatically reduced as GRP78 is depleted from PCs. This is not a cell-type-specific or cell death-associated observation, as this was also observed in GRP78-depleted viable prostate epithelial cells in another *Grp78* conditional knockout model.¹⁷ Although we cannot exclude the possibility that PCs without GRP78 result in a dramatic decrease in the cytosolic protein substrates for ubiquitination, the same cells show robust levels of GRP94,

PDI, calnexin, GADD34, CHOP, eIF2 α , calbindin, neurofilament and β -actin. A plausible explanation is that GRP78 is required for ER protein retrotranslocation to be ubiquitinated in the cytosol. In yeast, GRP78 facilitates the export of mutant proteins to the cytosol for degradation,³³ similarly, in mammalian cells, GRP78 stably binds to unfolded proteins and delivers them to the ERAD.³⁴ The molecular mechanism of GRP78 involvement is further clarified through the recent discovery that EDEM, ERdj5 and GRP78 form a functionally important ERAD complex.³⁵ ERdj5 functions as a disulfide reductase, which cleaves the disulfide bonds of misfolded proteins. ERdj5 binds to the ATP-bound form of GRP78 and activates ATP hydrolysis of GRP78, resulting in dissociation of GRP78 from ERdj5. In turn, the ADP-bound form of GRP78 binds protein substrates and holds them in a dislocation-competent state until they are transferred to the retrotranslocation channel in the ER membrane for export to the

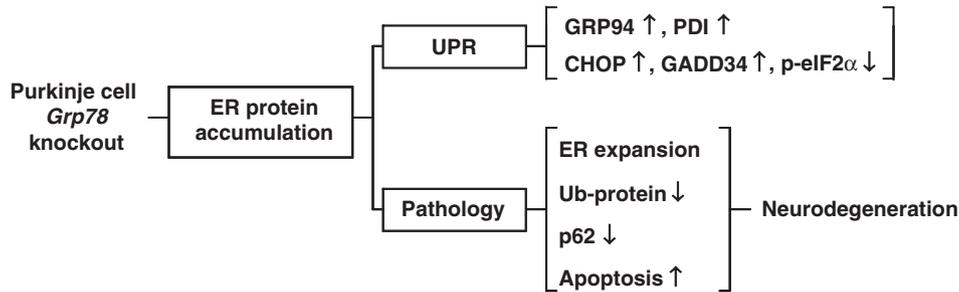


Figure 8 Summary model of modulation of the UPR and pathology resulting from Grp78 conditional knockout in PCs. GRP78 depletion in PCs leads to ER protein accumulation, abnormal ER expansion, block of protein ubiquitination and activation of the UPR and apoptosis, leading to neurodegeneration

cytosol and subsequent ubiquitination and degradation. Consistent with this model, ultrastructural images of GRP78-null PCs show the accumulation of electron-dense material inside the expanded ER, but not outside the ER. Nonetheless, the tipsy mouse model does not allow us to test this mechanism biochemically. Thus, the requirement of GRP78 in protein ubiquitination remains to be determined. In addition, whether other functions of GRP78, such as binding of ER Ca^{2+} , blocking the activation of ER-associated proapoptotic factors such as BIK and caspase-7^{22,36,37} and its requirement for stress-induced autophagy,³¹ contribute to PC survival awaits further investigation. Besides the cerebellum, GRP78 is most likely to be important for other critical neuronal functions, as it is recently reported that knock-in mice expressing a mutant secreted form of GRP78 display disordered layer formation in the cerebral cortex and cerebellum, resulting in a neurological phenotype of *reeler*-mutant-like malformation,³⁸ which is linked to mental disorders.³⁹ There is also accumulating evidence for age-dependent decrease in GRP78 expression or activity in specific organs and tissues, including the brain, suggesting that aging defects may result from GRP78 depletion in senescent cells.⁴⁰

In summary, our studies establish *Grp78* floxed mice as a valuable tool for studying the physiological role of GRP78 and the UPR in specific organs, as exemplified for the creation of tipsy mice. The latter differs from other models of PC death, as only PCs, but not other neurons, are directly targeted. Considering that PCs are a part of the core circuitry in the motor learning process, tipsy mice provide a unique model for behavioral studies of PC-specific function. Furthermore, the accelerated cerebellar degeneration in these mice will facilitate the development of therapeutic agents combating ER dysfunction in neurodegeneration and other diseases. Another exciting preclinical application is that this mouse model will provide an accelerated experimental system to access stem cell transplantation for the rescue of neurological phenotypes.

Materials and Methods

Generation of PC-specific and prostate-specific *Grp78* conditional knockout mice. Detailed information on the generation of *Grp78* F/F, F/- mice and prostate-specific *Grp78* conditional knockout mice was described previously.¹⁷ In brief, *Grp78* T/+ mice¹¹ carrying the targeted allele (T) were mated with *E11A-Cre* transgenic mice, and generated offspring carrying the floxed (F) or knockout (-) allele (Figure 1). PC-specific Cre (pc-Cre) transgenic

mice (line L7Cre-2) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA).¹⁸ *Grp78* F/-; pc-Cre mice were generated through breeding between *Grp78* F/- mice and PC-specific Cre transgenic mice. All protocols for animal use and killing were reviewed and approved by the University of Southern California Institutional Animal Care and Use Committee.

Primary antibodies. Primary antibodies used include the following: rabbit anti-GRP78 (H129) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-cleaved caspase-3 (Asp175) and phospho-eIF2 α (Ser51) (Cell Signaling, Danvers, MA, USA), anti-GRP94 (SPA-851), PDI (SPA890) and calnexin (SPA-865) (Stressgen, Ann Arbor, MI, USA) and anti-p62 (SQSTM1) (BIOMOL, Plymouth Meeting, PA, USA), mouse anti-calbindin (Sigma-Aldrich, St. Louis, MO, USA), anti-CHOP (GADD153 B-3) and GADD34 (C-19) (Santa Cruz Biotechnology), anti-eIF2 α (L57A5) and ubiquitin (P4D1) (Cell Signaling) and anti-neurofilament (nonphosphorylated) (SMI-32) (Sternberger Monoclonals, Baltimore, MD, USA).

Histological, immunohistochemical and immunofluorescence staining analyses. Mouse brains were exposed by removing the cranial bones and were fixed *in situ* overnight in 10% buffered formalin. The entire brain was then removed from the skull and cut midsagittally before being embedded in paraffin using standard protocols. Immunohistochemical and immunofluorescence staining were carried out as described previously.¹⁹ In brief, the Vectastain Elite avidin-biotin complex kit (Vector Laboratories, Burlingame, CA, USA) was used for immunohistochemistry. After antigen retrieval with retrivagen A (pH 6.0) (BD Pharmingen, San Jose, CA, USA), paraffin sections were incubated with primary antibodies in blocking solution (1.5% serum in PBS) at 4 °C overnight with antibodies against GRP78 (1:100), calbindin (1:100), cleaved caspase-3 (1:50), GRP94 (1:200), PDI (1:300), CHOP (1:50), eIF2 α (1:50), phospho-eIF2 α (Ser51) (1:50), ubiquitin (1:200) and p62 (1:1000). For immunofluorescence staining, paraffin sections were incubated with primary antibodies including calbindin (1:100), GADD34 (1:200), ubiquitin (1:100) and GRP78 (1:100), calnexin (1:200) and neurofilament (1:100) at 4 °C overnight. For fluorescent detection of calbindin, GADD34, ubiquitin and neurofilament, Alexa Fluor 488-conjugated goat anti-mouse IgG (1:200) (Invitrogen, Carlsbad, CA, USA) was used. For fluorescent detection of GRP78 and calnexin, Alexa Fluor 594-conjugated goat anti-rabbit IgG (1:200) (Invitrogen) was used. TUNEL assay was performed using the Peroxidase *In situ* Apoptosis Detection Kit (Chemicon International, Billerica, MD, USA).

Motor coordination tests. The rota rod test and footprint test were performed as previously described.⁵ In brief, for the rota rod test, mice were placed on a rod rotating at 30 r.p.m., and the time taken for them to fall from the rod was measured. If a mouse stayed on the rod longer than the 2 min trial, a time of 120 s was recorded. For the footprint test, the forelimbs and hindlimbs of mice were painted as blue and red with nontoxic ink, respectively. Their paw placement was then recorded using a narrow tunnel (10 cm wide, 40 cm long and 10 cm high) with white paper at the bottom.

Electron microscopy and immunoEM analysis. For regular EM, mouse brain tissues were dissected from the skull and immersion fixed in half strength Karnovsky's fixative (2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4) for 24 h at 4 °C. Tissues were postfixed in 1% osmium tetroxide for 2 h on ice. Samples were dehydrated in graded alcohol and

infiltrated with Eponate (Ted Pella, Redding, CA). Ultrathin sections were cut at 70 nm and stained with uranyl acetate and lead citrate. For immunoEM labeling, mouse brain tissues were dissected from the skull and immersion fixed in fixative (2% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer) for 24 h at 4 °C. Tissues were infiltrated with LR White acrylic resin (Ted Pella). After polymerization, ultrathin sections were cut at 70 nm. After incubation with blocking solution (1% BSA and 0.1% Triton X-100 in PBS) for 30 min, sections were stained overnight with anti-calnexin antibody (1 : 40) at 4 °C, followed by 1 h incubation with 15 nm gold-conjugated goat anti-rabbit antibody (1 : 40) (Ted Pella). Sections were counterstained with uranyl acetate. All sections were examined using a JEOL JEM 2100 electron microscope (Peabody, MA) and photographed using an Orius SC1000B Gatan digital camera (Pleasanton, CA).

Body weight and food consumption measurements. Fasting body weights of the mice were measured weekly from 3 to 10 weeks of age. Food consumption was measured for seven successive days at 3 and 9 weeks of age.

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