XBP-1 deficiency in the nervous system protects against amyotrophic lateral sclerosis by increasing autophagy

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Mutations in superoxide dismutase-1 (SOD1) cause familial amyotrophic lateral sclerosis (fALS). Recent evidence implicates adaptive responses to endoplasmic reticulum (ER) stress in the disease process via a pathway known as the unfolded protein response (UPR). Here, we investigated the contribution to fALS of X-box-binding protein-1 (XBP-1), a key UPR transcription factor that regulates genes involved in protein folding and quality control. Despite expectations that XBP-1 deficiency would enhance the pathogenesis of mutant SOD1, we observed a dramatic decrease in its toxicity due to an enhanced clearance of mutant SOD1 aggregates by macroautophagy, a cellular pathway involved in lysosome-mediated protein degradation. To validate these observations in vivo, we generated mutant SOD1 transgenic mice with specific deletion of XBP-1 in the nervous system. XBP-1-deficient mice were more resistant to developing disease, correlating with increased levels of autophagy in motoneurons and reduced accumulation of mutant SOD1 aggregates in the spinal cord. Post-mortem spinal cord samples from patients with sporadic ALS and fALS displayed a marked activation of both the UPR and autophagy. Our results reveal a new function of XBP-1 in the control of autophagy and indicate critical cross-talk between these two signaling pathways that can provide protection against neurodegeneration.

[Keywords: Amyotrophic lateral sclerosis; unfolded protein response; endoplasmic reticulum stress; XBP-1; autophagy]

Supplemental material is available at http://www.genesdev.org.

Received June 10, 2009; revised version accepted August 19, 2009.
to developing the disease. This phenotype was associated with a decrease in the levels of mutant SOD1 inclusions in these cells. A nearly 50% reduction in the number of cells harboring SOD1<sup>GW</sup> and SOD1<sup>G85R</sup> intracellular inclusions was detected in shXBP-1 and shIRE1 cells (Fig. 1D). Similar results were obtained when XBP-1 and XBP-1 knockdowns were performed in Neuro2a cells (data not shown). As control, to monitor the efficiency of protein expression/cell transfection, SOD1<sup>WT</sup>-EGFP was expressed in shXBP-1, shIRE1, and shControl cells, and then quantified by FACS (Supplemental Fig. S1E).

Consistent with a decrease in the levels of mutant SOD1 misfolding, shXBP-1 cells displayed increased survival after SOD1<sup>G85R</sup> expression as measured by monitoring mitochondrial activity with the MTT assay (Fig. 1F). We also investigated the effects of XBP-1 loss of function by transfection of an XBP-1 expression vector with SOD1<sup>G93A</sup> or SOD1<sup>G85R</sup> constructs. We observed the increased aggregation of mutant SOD1 proteins and the modest increase in the number of cells harboring SOD1<sup>G93A</sup> and SOD1<sup>G85R</sup> inclusions in shXBP-1 cells (Fig. 1D). These results suggest that XBP-1 plays an important role in the clearance of mutant SOD1 in vivo.

Results

Knocking down XBP-1 and IRE1α decreases mutant SOD1 aggregation and toxicity

To define the role of the UPR in SOD1 pathogenesis, we reduced the expression levels of major UPR components in the NSC34 motoneuron cell line (Hetz et al. 2007) using lentiviral delivery of shRNAs. Targeted genes included IRE1α, XBP-1, activating transcription factor-6α (ATF6α) and ATF4. In addition to XBP-1, ATF6 and ATF4 constitute two parallel signaling branches of the UPR that regulate distinct transcriptional responses under ER stress (Ron and Walter 2007). To monitor SOD1 misfolding in these cells, we transiently expressed human SOD1<sup>WT</sup> or the mutants SOD1<sup>G93A</sup> and SOD1<sup>G85R</sup> as EGFP fusion proteins and examined the accumulation of intracellular SOD1 inclusions by fluorescent microscopy or SOD1 aggregation by Western blot analysis. In agreement with the known role of ATF4 and ATF6α in the transcriptional control of ER chaperones, knocking down these UPR components increased mutant SOD1 aggregation (Supplemental Fig. S1A–C). We verified functional knockdown of XBP-1 as evidenced by the significant reduction in expression of XBP-1 protein and many XBP1-target genes (Fig. 1A,C). Similarly, IRE1α knockdown reduced the levels of XBP-1 mRNA splicing and XBP-1 expression under ER stress conditions (Fig. 1B). Surprisingly, a drastic reduction in the generation of high-molecular-weight and detergent-insoluble SOD1 species was observed in XBP-1 and IRE1α knockdown NSC34 cells (Fig. 1E, Supplemental Fig. S1D for total extracts). To complement these observations, we monitored the accumulation of SOD1 inclusions in these cells. A nearly 50% reduction in the number of cells harboring SOD1<sup>GW</sup> and SOD1<sup>G85R</sup> intracellular inclusions was detected in shXBP-1 and shIRE1 cells (Fig. 1D). Similar results were obtained when IRE1α and XBP-1 knockdowns were performed in Neuro2a cells (data not shown). As control, to monitor the efficiency of protein expression/cell transfection, SOD1<sup>WT</sup>-EGFP was expressed in shXBP-1, shIRE1, and shControl cells, and then quantified by FACS (Supplemental Fig. S1E).

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SOD1 and augmented generation of intracellular inclusions (Fig. 1G). Taken together, these results revealed an unexpected role of the IRE1α/XBP-1 axis of the UPR on SOD1 pathogenesis.

Autophagy-mediated degradation of mutant SOD1 in XBP-1-deficient motoneurons

Diminished SOD1 aggregation in XBP-1 knockdown NSC34 cells might be explained by the up-regulation of protein degradation pathways involved in mutant SOD1 clearance. Both the proteasome and macroautophagy (referred to here as autophagy) [Rubinsztein 2006; Mizushima et al. 2008] pathways have been shown to mediate mutant SOD1 degradation in vitro [Kabuta et al. 2006]. To define the contribution of these pathways to SOD1 clearance, we treated shRNA NSC34 cells with proteasome (MG-132) or phosphatidylinositol-3 (PI3) kinase inhibitors (3-methyladenine [3-MA] and Wortmannin), which block an early step controlling autophagosome
formation [Levine and Kroemer 2008; Mizushima et al. 2008], and thus inhibit autophagy. Blocking PI3 kinases resulted in more SOD1 aggregation than did proteasome inhibition, with recovery of mutant SOD1 aggregation in knockdown cells [Fig. 2A]. In agreement with these results, no changes in basal proteasomal activity were observed after knocking down XBP-1 [Supplemental Fig. S2A].

Autophagosomes fuse with lysosomes, forming autophagolysosomes where their content is degraded (Rubinsztein 2006; Mizushima et al. 2008). In order to study the role of the lysosomal compartment in the degradation of mutant SOD1, we first analyzed its possible localization at the lysosome. A clear colocalization between SOD1 mutant inclusions and acidic compartments was observed in NSC34 cells when compared with wild-type SOD1 [Fig. 2B, Supplemental Fig. S2B]. To measure the functional degradation of mutant SOD1 by the lysosomal pathway, we treated shXBP-1 cells with a cocktail of lysosomal inhibitors [bafilomycin A1 and the protease inhibitors pepstatin and E64d]. Using this approach, we observed an enhanced accumulation of SOD1 aggregates and inclusions in shXBP-1 cells after inhibiting lysosomal activity [Fig. 2C].

We extended our results by knocking down ATG5, a major autophagy regulator in the nervous system [Hara et al. 2006]. We transduced shXBP-1 cells with shRNA lentiviruses against the atg5 mRNA, which reduced its mRNA levels by ~70% [Fig. 2D]. A significant increase in the levels of mutant SOD1 aggregation was observed in shXBP-1 cells when ATG5 expression was knocked down, reverting the phenotype of XBP-1 deficiency.

A shorter exposure [low expo.] of the same Western blot shows monomeric SOD1. HSP90 levels were monitored as loading control. Data are representative of four determinations.
Similar results were obtained when we targeted the expression of Beclin-1/ATG6, the first identified mammalian gene product shown to regulate autophagy (Liang et al. 1999; for review, see Mizushima et al. 2008), in shXB1-1 cells (Supplemental Fig. S2C). Thus, our results indicate that XBP-1 deficiency increases mutant SOD1 clearance due to autophagy-mediated degradation.

**Targeting XBP-1 up-regulates basal autophagy activity**

Based on the previous results, we then investigated the possible role of XBP-1 in the regulation of autophagy. LC3 (also known as ATG8 in yeast) is a commonly used marker of autophagy that localizes specifically to autophagosomes (Klionsky et al. 2008). Using LC3-EGFP fusion to determine autophagosome content, we observed a clear increase in the number of shXB1-1 cells containing autophagosomes compared with control cells (Fig. 3A). As control for the assay, shXB1-1 cells were treated with 3-MA, which drastically reduced the amount of LC3-positive vesicles to a similar level as shControl cells (Fig. 3A). We complemented these studies by measuring the activity of lysosomes using DQ-BSA, a dye that stains active proteolysis at the lysosome, and observed a significant increase in the content of active lysosomes in XBP-1 knockdown motoneurons (Fig. 3B). Similarly, increased lysosome content was detected after visualization of shXB1-1 cells with lysotracker, acridine orange staining, or electron microscopy (Supplemental Fig. S2D–F).

To determine whether the presence of LC3-positive vacuoles is related to augmented autophagy activity, we first measured the basal levels of the endogenous lipidated LC3-II form by Western blot in shControl (M) or shXB1-1 (X) cells under resting conditions. To monitor the flux of LC3 through the autophagy pathway, experiments were performed in the presence or absence of a lysosome inhibitor cocktail (Lys. Inh.) containing 200 nM bafilomycin A1, 10 μg/mL pepstatin, and 10 μg/mL E64d for the indicated time points. As control, in shXB1-1 NSC34 cells, ATG5 was also knocked down with shRNAs and LC3-II levels were monitored by Western blot. shControl and shXB1-1 cells were transiently transfected with a tandem monomeric LC3-RFP-GFP construct to monitor the active flux of LC3 through the autophagy pathway. After 48 h, LC3-positive dots were visualized by fluorescent microscopy in the red and green channels and the ratio between the number of red dots (autophagolysosomes, acidic compartment) versus colocalized yellow dots (representing autophagosomes) per cell was determined. Mean and standard deviation are presented. Representative overlapped fluorescent images are presented. Bar, 10 μm. (E,F) shControl, shXB1-1, and shIRE1α cells were maintained in rich culture medium or incubated in EBSS buffer for 2 h, and mitochondrial metabolism was determined using the MTT assay (E) or cell viability was monitored by propidium iodide staining (F). Overlapping phase contrast and propidium iodide staining images are shown. Average and standard deviation are presented of three to five determinations.
rather than to decreased lysosomal fusion/degradative activity, we monitored the flow of LC3 through the autophagy pathway. The level of endogenous LC3-II (the active phosphatidylethanolamine-conjugated form) was measured under resting conditions in shXBP-1 and control cells in the presence or absence of lysosomal inhibitors. We observed a clear increase in the expression of LC3-II in shXBP-1 cells when compared with control cells that was reverted by knocking down ATG5 [Fig. 3C]. Interestingly, LC3-II levels were further augmented by blocking lysosomal activity [Fig. 3C], indicating elevated autophagy activity and LC3 flux in shXBP-1 cells. These data were also corroborated by expressing a tandem monomeric RFP-GFP-tagged LC3, where the LC3 flux into the lysosomal acidic compartment can be followed in living cells in the absence of drug treatment [Fig. 3D; Klionsky et al. 2008].

Since autophagy was described originally as a survival pathway under limiting nutrient conditions, we tested the cellular consequence of increased basal autophagy in IRE1α- and XBP-1-deficient NSC34 cells by analyzing their susceptibility to nutrient starvation (assessment of preconditioning effect). Using two independent methods, we observed that shXBP-1 and shIRE1 cells were more resistant to starvation-induced cell death induced by incubating cells in Earle’s balanced salt solution (EBSS) buffer [Fig. 3E,F]. Together, these results indicate that functional autophagy is overactive in cells where IRE1α signaling is impaired.

ER-associated degradation (ERAD) impairment increases basal autophagy

Through ERAD, misfolded proteins accumulated at the ER are retrotranslocated to the cytosol for degradation by the proteasome, and ERAD-related genes are a major target of XBP-1s [Lee et al. 2003]. Autophagy has been suggested to act as a second ERAD pathway for degradation of ER-located misfolded proteins [Fujita et al. 2007]. Because proteasome impairment has been shown to trigger autophagy [Ding et al. 2007; Pandey et al. 2007; Kaganovich et al. 2008], we hypothesized that XBP-1 deficiency may affect ERAD and other related processes, leading to increased basal levels of misfolded proteins at the ER, activating autophagy as a survival mechanism. To determine the effects of XBP-1 on ERAD activity in motoneurons, we first monitored the levels of a classical ERAD substrate, CD3-δ-YFP [Lerner et al. 2007], in shXBP-1 cells under resting conditions. To determine the flow of CD3-δ-YFP through the ERAD pathway for proteasome-mediated degradation, experiments were performed after inhibition of translation by cycloheximide treatment in the presence or absence of proteasome inhibitors. Western blot analysis of CD3-δ-YFP expressing cells revealed a marked decrease in its degradation in shXBP-1 cells when compared with control cells [Fig. 4A]. To test the possible involvement of ERAD-related XBP-1 target genes in the up-regulation of autophagy, we knocked down EDEM1, a key protein in ERAD [Molinari et al. 2003] that is specifically up-regulated by XBP-1 under ER stress conditions [Lee et al. 2003]. Interestingly, decreasing EDEM1 levels in NSC34 cells recapitulated the phenotype of XBP-1 silencing, with reduced accumulation of SOD1G85R detergent-insoluble species [Fig. 4B]. In addition, elevated numbers of LC3-labeled autophagosomes were observed in these cells [Fig. 4C]. These results suggest that the protective effects of XBP-1 deficiency on SOD1 pathogenesis are related to its essential regulation of ERAD function.
XBP-1 deficiency prolongs the life span of a mutant SOD1 transgenic mouse

To establish the role of XBP-1 in fALS in vivo, we first monitored the levels of ER stress markers in the spinal cord of SOD1\textsuperscript{G86R} or mouse SOD1\textsuperscript{G86R} (the equivalent to human SOD1\textsuperscript{G86R} mutation) transgenic mice. In agreement with previous reports, we observed the up-regulation of PDI, Grp78/BiP, and CHOP in symptomatic animals [Supplemental Fig. S3A]. More importantly, XBP-1 mRNA splicing was observed in the SOD1\textsuperscript{G86R} transgenic mice (Fig. 5A), which correlated with the up-regulation of its target genes edem [Fig. 5B], herp, erdj4, wfs-1, sec61, grp58, and other UPR-related genes [Supplemental Fig. S3B].

To establish the contribution of XBP-1 to fALS in vivo, we generated an XBP-1 conditional knockout model in which xbp-1 was deleted in the nervous system using the Nestin-Cre system [XBP-1\textsuperscript{Nes\textsuperscript{-/-}}] (Hetz et al. 2008). These mice developed normally and did not show any overt spontaneous disease phenotype as we described recently [Hetz et al. 2008]. We cross-bred XBP-1\textsuperscript{Nes\textsuperscript{-/-}} mice with SOD1\textsuperscript{G86R} transgenic mice to evaluate the role of XBP-1 in animal survival. Consistent with our cellular studies, XBP-1 deficiency resulted in an average overall increase in life span of 10 d in SOD1\textsuperscript{G86R} mice [Supplemental Fig. S4A]. Interestingly, analysis by gender revealed that this was entirely due to the effect of XBP1 deficiency in female animals. XBP-1 deficiency in females resulted in a highly significant increase in life span of 22 d ($P = 0.0019$), with an average survival of 110 and 132 d for XBP-1\textsuperscript{WT}, SOD1\textsuperscript{G86R} and XBP-1\textsuperscript{Nes\textsuperscript{-/-}}, SOD1\textsuperscript{G86R} mice, respectively [Fig. 5C]. Analysis of male animals did not show a significant effect on life span [Supplemental Fig. S4B]. Life span extension was associated with a delay in the disease onset in XBP-1\textsuperscript{Nes\textsuperscript{-/-}} mice, but the duration of the symptomatic phase of the disease was not affected by XBP-1 deficiency or gender when compared with control animals [Supplemental Fig. S4C,D]. The increased life span in females correlated with a 30% decrease in apoptosis in the ventral horn of the spinal cord of XBP-1\textsuperscript{Nes\textsuperscript{-/-}}, SOD1\textsuperscript{G86R} mice compared with control animals ($P = 0.03$) [Fig. 5D]. Thus, despite our initial prediction that impairment of the IRE1a/XBP-1-dependent adaptive response would accelerate neuronal dysfunction and disease severity, therefore decreasing the life span of fALS mouse models, we observed significant protection against disease in female animals.

![Figure 5](image-url)

**Figure 5.** XBP-1 deficiency in the nervous system prolongs life span of mutant SOD1 transgenic mice and decreases the levels of apoptosis. [A] The levels of XBP-1 mRNA splicing [XBP-1s] and nonspliced [XBP-1u/s] were determined in the spinal cord of three symptomatic SOD1\textsuperscript{G86R} transgenic mice by RT–PCR. [B] The mRNA level of the XBP-1 target gene edem1 was analyzed by real-time PCR in total cDNA obtained from the spinal cord of five SOD1\textsuperscript{G86R} or four littermate control mice. All samples were normalized to $\beta$-actin levels. Average and standard deviation are presented. P-value was calculated using Student’s t-test. [C] XBP-1\textsuperscript{Nes\textsuperscript{-/-}} ($N = 7$) and control wild-type ($N = 9$) mice were bred onto SOD1\textsuperscript{G86R} transgenic mice and survival was evaluated in female animals. P-value was calculated with Kaplan-Meier statistics. [D] TUNEL-positive cells in the right half of the ventral horn were quantified in a total of five animals per group in mice at the late stage of the disease. Average and standard deviation are presented. Indicated P-value was calculated using Student’s t-test.

XBP-1 deficiency increases autophagy and SOD1 degradation in vivo

To test whether XBP-1 deficiency alters autophagy levels in fALS in vivo, we monitored the number of neurons containing autophagosomes in the ventral horn of the spinal cord. A marked increase of neurons containing LC3-labeled autophagosomes was observed in SOD1\textsuperscript{G86R}/XBP-1\textsuperscript{Nes\textsuperscript{-/-}} mice when compared with control animals [Fig. 6A]. Histological quantification of different animals revealed that $\sim$50% of neurons in the ventral horn contained LC3-positive autophagosomes ($P = 0.005$) [Fig. 6A]. In contrast, SOD1\textsuperscript{G86R} mice on a wild-type background showed only a slight increase in the number of autophagosomes in some animals when compared with nontransgenic animals, totaling, on average, $\sim$15% of neurons containing LC3-positive vesicles [Fig. 6A]. Of note, the majority of LC3-positive cells were positive for NeuN staining ($\geq$90%) in the ventral horn [Supplemental Fig. S5A]. In agreement with this observation, astrocytes were, in general, negative for LC3-positive dots [Fig. 6B], indicating a specific contribution of XBP-1 deficiency to the enhancement of autophagy in neurons. In addition, although SOD1\textsuperscript{G86R} transgenic mice show clear signs of glial activation, this phenomenon was not drastically affected by XBP-1 deficiency [Supplemental Fig. S6].

Analysis of lysosomal content by LAMP-2 staining revealed a higher content in motoneurons of SOD1\textsuperscript{G86R}/XBP-1\textsuperscript{Nes\textsuperscript{-/-}} mice when compared with control mice [Fig. 6C]. Similar results were observed when autophagosomal structures were visualized by electron microscopy (see Supplemental Fig. S5B). In addition, we measured the
expression of LC3-II in the spinal cord of symptomatic SOD1<sup>G86R</sup> mice bred onto a wild-type or XBP-1<sup>Nes</sup>/C0 background. In agreement with the histological characterization, accumulation of LC3-II was only evident by Western blot analysis in XBP-1<sup>Nes</sup>/C0–SOD1<sup>G86R</sup> mice (Fig. 6D), which may be related to the low abundance of autophagosomes in SOD1<sup>G86R</sup> control mice as visualized by histology, and would be diluted in a total tissue extract. Interestingly, Western blot analysis revealed induction of the autophagy regulator Beclin-1 in the XBP-1-sufficient SOD1<sup>G86R</sup> strain that was further increased in XBP-1<sup>Nes</sup>/C0–SOD1<sup>G86R</sup> animals (Fig. 6D). Although alterations in Beclin-1 levels are not usually used as an indication of autophagy, changes in Beclin-1 expression have been reported to correlate well with autophagy levels in animal models of brain ischemia and injury (Diskin et al. 2005; Carloni et al. 2008; Rami et al. 2008), and autophagy-mediated clearance of aggregate-prone proteins in models of Huntington’s and Alzheimer’s disease (Shibata et al. 2006; Pickford et al. 2008). Overall, our results indicate that the slight increase in autophagy in SOD1 mutant spinal cords described previously (Morimot et al. 2007) is significantly enhanced when XBP-1 is ablated in the nervous system.

To monitor the active engulfment of SOD1 aggregates by autophagy in the spinal cord of XBP-1<sup>Nes</sup>/C0 mice, we performed SOD1 immunogold staining and electron microscopy analysis. We were able to visualize SOD1 inside autophagosomes using this method (Fig. 7A). In addition, we detected a colocalization of SOD1-positive dots (red) with neurons [NeuN, blue] or astrocytes [GFAP, green] was analyzed in the spinal cord of a mSOD1<sup>G86R</sup> transgenic mice using immunofluorescence. A merged picture is presented where white arrows indicate astrocytes and red arrows indicate neurons. A negative control of staining without primary antibodies is presented. Bar, 20 μm. (C) Lysosomes were visualized in the samples in A after LAMP-2 staining (red). Colocalization with motoneurons was evaluated after ChAT [green] staining [white arrow]. Total cells were stained with Hoechst [nucleus, blue]. Quantification of LAMP-2-positive motoneurons is indicated in the inset. Bar, 20 μm.

Figure 6. XBP-1 deficiency increases autophagy levels in neurons of SOD1<sup>G86R</sup> transgenic mice. (A) Autophagosomes were directly observed in the spinal cord of control of mSOD1<sup>G86R</sup> transgenic mice on an XBP-1<sup>WT</sup> or XBP-1<sup>Nes</sup>/C0 background by immunofluorescence using an anti-LC3 antibody [green]. Neurons were costained with an anti-NeuN antibody [red] and with Hoechst [nucleus, blue]. Images are representative of the analysis of five different animals per group of ~125–130 d of age. [Right panel] Quantification of the percentage of NeuN-positive cells containing LC3-positive vacuoles. Values represent average and standard deviation. P-value was calculated using Student’s t-test. Bar, 10 μm. (B) The colocalization between LC3-positive dots [red] with neurons [NeuN, blue] or astrocytes [GFAP, green] was analyzed in the spinal cord of mSOD1<sup>G86R</sup> transgenic mice using immunofluorescence. A merged picture is presented where white arrows indicate astrocytes and red arrows indicate neurons. A negative control of staining without primary antibodies is presented. Bar, 20 μm. (C) Lysosomes were visualized in the samples in A after LAMP-2 staining [red]. Colocalization with motoneurons was evaluated after ChAT [green] staining [white arrow]. Total cells were stained with Hoechst [nucleus, blue]. Quantification of LAMP-2-positive motoneurons is indicated in the inset. Bar, 20 μm.

(D) Beclin-1, LC3, and Hsp90 expression were determined in spinal cord protein extracts from symptomatic mSOD1<sup>G86R</sup> transgenic or control mice by Western blot. LC3-II form is indicated. Two representative animals are shown per group.
were not significantly affected in male XBP-1Nes/−/− SOD1G86R animals compared with control mice [Supplemental Fig. S7A,B]. Taken together, these data indicate that XBP-1 deficiency in female mice leads to increased autophagy, associated with augmented mutant SOD1 clearance and prolonged life span.

Up-regulation of autophagy in the spinal cord of sALS and fALS cases

Finally, to characterize the role of autophagy in sALS and fALS, we determined the levels of LC3-I, LC3-II, BECLIN-1, and the ATG5–ATG12 complex in human post-mortem spinal cord samples. A marked induction of autophagy markers was observed in the majority of samples analyzed when compared with age-matched, healthy control subjects [Fig. 7D]. Interestingly, the relative levels of BECLIN-1 induction correlated well with increased amounts of polyubiquitinated proteins [Fig. 7D]. Two recent reports (Ilieva et al. 2007; Atkin et al. 2008) indicated that signs of ER stress are observed in the spinal cord of sALS post-mortem samples. To determine whether XBP-1 and the UPR are active in sALS, we analyzed the expression levels of different UPR markers in post-mortem spinal cord samples from sALS patients. There was marked expression of XBP-1s and ATF4 in sALS cases, up to a 4.5-fold increase, in addition to the ER chaperone Grp58 [Supplemental Fig. S8]. Consistent with these results, increased expression of the XBP-1s target EDEM1 was observed in both sALS and fALS cases [Fig. 7D]. These data corroborate previous findings suggesting that ER stress is observed in sALS patients, and suggest an active engagement of the UPR and autophagy in ALS.

Discussion

A common feature of many neurodegenerative diseases is the accumulation of misfolded proteins in the brain, affecting cognitive and motor functions. Increased expression of ER stress markers is observed in post-mortem brain tissues from patients affected with diseases such as Parkinson’s disease, ALS, Alzheimer’s disease, and Creutzfeldt-Jacob’s disease, in addition to mouse models
IRE1 deficiency cells, uncovering an unexpected function of the XBP-1 axis of the UPR. This phenotype was explained by the up-regulation of autophagy in XBP-1-deficient cells, uncovering an unexpected function of the IRE1α/XBP-1 axis of the UPR.

The occurrence of ER stress is associated with disease progression and motoneuron degeneration in ALS mouse models, and occurs during early presymptomatic stages (Saxena et al. 2009). Activation of the three major UPR stress pathways is observed in the spinal cord of mutant SOD1 transgenic mice (Atkin et al. 2006; Kikuchi et al. 2006). Interestingly, mutations in ALS8/VAPB, a gene known to affect ATF6 and XBP-1 signaling (Gkogkas et al. 2008), increasing the susceptibility of cells to ER stress-mediated apoptosis (Suzuki et al. 2009). More importantly, analysis of post-mortem samples derived from sALS patients demonstrated up-regulation of UPR components (Li et al. 2007, Atkin et al. 2008). Our results confirmed these observations and further demonstrated for the first time up-regulation of the UPR transcription factors ATF4 and XBP-1 in tissue from human sALS patients. Overall, these studies suggest that ER stress and protein misfolding is a common feature of sALS and different forms of fALS.

To address the function of XBP-1 in fALS in vivo, we studied the susceptibility of mice with selective deficiency of XBP-1 in the nervous system to experimental ALS. Targeting XBP-1 in the nervous system increased life span and motoneuron survival of mutant SOD1 transgenic mice, despite initial predictions that XBP-1 ablation, as these cells are known to contribute to ALS pathogenesis (Pasinelli and Brown 2006). Of note, a recent study demonstrated that neuronal expression of mutant SOD1 in transgenic mice is sufficient to cause motoneuron degeneration and paralysis (Jaarsma et al. 2008). Conversely, we observed that most of the cells displaying LC3-labeled autophagosomes in the ventral horn of XBP-1−/−/SOD1G85R mice were neurons and not astrocytes, suggesting a specific effect of XBP-1 deficiency on autophagy levels in neurons.

The effect of XBP-1 ablation was more evident in females, suggesting that the protective consequences of autophagy may be influenced by gender. A recent report also indicated that targeting BECLIN-1-dependent autophagy in the context of an Alzheimer’s disease model had beneficial effects only in female and not in male animals [Pickford et al. 2008], suggesting that autophagy levels are influenced by gender. We obtained data supporting this hypothesis by monitoring the levels of LC3-II conversion in male and female mice using a classical model of autophagy in which nutrient starvation triggers the process in muscle tissue [Supplemental Fig. S7C; Mizushima et al. 2004]. Interestingly, the incidence of human ALS is higher in males than females, and other reports have detected marked differences between genders in SOD1 transgenic mice (for examples, see Lepore et al. 2007; Stam et al. 2008), although the molecular basis of these gender differences is not understood. Along this line, we observed intrinsic differences in our SOD1G85R transgenic mice, where the accumulation of SOD1 aggregates was higher in males than females despite similar mRNA levels between genders [Supplemental Fig. S7D,E].

In contrast to our results, a recent report indicated that treatment of mutant SOD1 transgenic mice with salubriol [Saxena et al. 2009], a small molecule that augments eIF2α phosphorylation with concomitant ATF4 up-regulation, significantly delays disease onset, increasing life span. These results are consistent with the effects of ATF4 knockdown in mutant SOD1 aggregation. Taken together with the current study, an intriguing scenario emerges in which the contribution of ER stress to protein misfolding disorders is more complex than previously anticipated, since each UPR signaling branch may have distinct effects on the cellular responses to mutant SOD1 protein misfolding.

In addition to its role as a survival pathway under conditions of nutrient starvation, autophagy is critical for the maintenance of neuronal homeostasis and contributes to basal elimination of misfolded proteins, and brain-specific ablation of essential autophagy-related genes results in spontaneous neurodegeneration [Hara et al. 2006; Komatsu et al. 2006]. In addition, pharmacological activation of autophagy has proven beneficial in different models of neurodegeneration [for reviews, see Rubinsztein 2006; Matus et al. 2008]. Our analysis of the XBP-1−/− ALS model strain has uncovered a heretofore unappreciated cross-talk between autophagy and the UPR in the nervous system, where homeostatic balance related to XBP-1 deficiency lead to increased autophagy, possibly due to ERAD impairment. ERAD is an essential process controlled by XBP-1 that decreases the unfolded protein load at the ER. However, recent data indicate that disease-related protein aggregates linked to neurodegeneration are poorly degraded by the proteasome [Rubinsztein 2006], and in some cases can even block ERAD, triggering ER stress (Duenwald and Lindquist 2008; Nishitoh et al. 2008).

The functional connection between the UPR and autophagy may explain the lack of lethality or spontaneous disease in XBP-1−/− mice (model in Supplemental
Fig. S9). Because many aggregate-prone proteins, such as SOD1, cause disease by a gain-of-function mechanism, therapeutic strategies that reduce the levels of such proteins by increasing autophagy may be beneficial. In fact, we observed a clear induction of several autophagy markers in ALS and iALS spinal cord samples, indicating that autophagy is a relevant cellular response in ALS. Our results uncover a new function of XBP-1 in vivo, and suggest that small molecules such as IRE1α inhibitors and chemical chaperones in addition to autophagy activators may be a feasible strategy for the treatment of protein folding disorders in the nervous system.

Materials and methods

Knockdown of UPR and autophagy components in motoneurons

We generated stable motoneuron cell lines with reduced levels of XBP-1, IRE1α, Beclin-1, and EDEM1 using methods described previously [Hetz et al. 2007] by targeting the respective mRNA with shRNA using the lentiviral expression vector pLKO.1 and puromycin selection.

Assays for mutant SOD1 aggregation and detection of intracellular inclusions

We developed assays using the transient expression of human SOD1WT and the mutants SOD1G93A and SOD1G85R as EGFP fusion proteins. SOD1 oligomers were visualized in total cell extracts prepared in RIPA buffer and sonication, and then analyzed by Western blot. Alternatively, nuclear cell lysates were prepared in 1% NP-40 in PBS containing protease inhibitors. After solubilization for 30 min on ice, cell nuclei were precipitated by centrifugation at 3000 rpm for 5 min and cell extracts were centrifuged at 10,000 g for 10 min to collect NP-40-soluble and -insoluble material. Pellets were resuspended in Western blot sampler buffer containing SDS.

Quantification of autophagy and cell viability

Different assays and control experiments were used to monitor autophagy-related processes following the recommendations and precautions described in Klionsky et al. (2008). Living cells were stained with 200 nM lysotracker or 600 nM Acridine orange for 45 min at 37 °C and 5% CO2. Alternatively, cells were loaded with DQ-BSA to monitor lysosomal activity as described previously [Klionsky et al. 2008]. Autophagy was monitored by analyzing LC3-positive dots or the levels of LC3-II by Western blot and its flux through the autophagosomal/lysosomal pathway by treating cells with a mix of 200 nM bafilomycin A1, 10 µg/mL pepstatin, and 10 µg/mL E64d. Alternatively, to monitor flux, we transiently expressed a tandem monomeric RFP-GFP-tagged LC3 [Klionsky et al. 2008]. Cell viability was monitored using the MTT assay or propidium iodide staining (Lisbona et al. 2009). Proteasomal or ERAD activity was monitored using the MTT assay or propidium iodide staining as described previously [Hetz et al. 2007, 2008].

Assays for mutant SOD1 aggregation and detection of intracellular inclusions

One centimeter of lumbar spinal cord tissue was collected and homogenized in RIPA buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 0.1% SDS, 0.5% DOC, 0.5% Triton X-100) containing a protease inhibitor cocktail (Roche) by sonication. Antibodies and dilutions used are described in the Supplemental Material.

Western blot analysis of spinal cord extracts

Electron microscopy studies and immunogold staining

Autophagosomes were also visualized by transmission electron microscopy as in Klionsky et al. (2008) and morphology was examined using standard methods [Eskelinen 2008]. Immunogold electron microscopy staining was performed as described previously [Court et al. 2008].

ALS human post-mortem spinal cord samples

Human post-mortem tissue from ALS and control subjects was obtained as frozen tissue from the Massachusetts General Hospital and then processed for biochemical analysis. The gender, genotype, age of death, and identity numbers are indicated in Supplemental Table 1.

Statistical analysis

Data were analyzed by Student’s t-test, two-way ANOVA, or Kaplan-Meier statistics. The GraphPad Prism 5 software was used for statistical analysis. Complete methodological details are described in the Supplemental Material.

Acknowledgments

We thank Drs. Fabio Martinon, Sebastian Bernales, and Sergio Lavandero for helpful discussion, and Benjamin Caballero and Sylvia Flores for technical assistance. We thank Dianne Anne-Marie Wills (MD) for organizing tissue transfer from the ALS tissue bank, Patricia Boya and Maria Luisa Colombo for advice about autophagy assays, and Drs. Noburo Mizushima and Tamotsu Yoshimori for providing LC3-EGFP and LC3-RFP-EGFP constructs. We are grateful to Drs. Julie Atkin and Bradley Turner for kindly providing expression vectors for mutant and wild-type SOD1-EGFP, and Dr. Nir Hacohen and The Broad Institute for providing shRNA lentiviral constructs. We thank Dr. Ron Kopito for providing the GFPu expression vector, and
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