

# Neuronal dystonin isoform 2 is a mediator of endoplasmic reticulum structure and function

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**ABSTRACT** Dystonin/Bpag1 is a cytoskeletal linker protein whose loss of function in dystonia musculorum (*dt*) mice results in hereditary sensory neuropathy. Although loss of expression of neuronal dystonin isoforms (dystonin-a1/dystonin-a2) is sufficient to cause *dt* pathogenesis, the diverging function of each isoform and what pathological mechanisms are activated upon their loss remains unclear. Here we show that *dt*<sup>27</sup> mice manifest ultrastructural defects at the endoplasmic reticulum (ER) in sensory neurons corresponding to in vivo induction of ER stress proteins. ER stress subsequently leads to sensory neurodegeneration through induction of a proapoptotic caspase cascade. *dt* sensory neurons display neurodegenerative pathologies, including Ca<sup>2+</sup> dyshomeostasis, unfolded protein response (UPR) induction, caspase activation, and apoptosis. Isoform-specific loss-of-function analysis attributes these neurodegenerative pathologies to specific loss of dystonin-a2. Inhibition of either UPR or caspase signaling promotes the viability of cells deficient in dystonin. This study provides insight into the mechanism of *dt* neuropathology and proposes a role for dystonin-a2 as a mediator of normal ER structure and function.

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## INTRODUCTION

The eukaryotic cytoskeleton comprises three cytoskeletal networks, including microtubules, actin microfilaments, and intermediate filaments. These filaments rely on cytoskeletal cross-linking proteins to facilitate their multifaceted functions, ranging from vesicular transport and maintenance of organelle integrity to mitosis (Fuchs and Karakesisoglou, 2001). As such, loss of cytoskeletal-linking proteins can have grave implications on cellular functioning and viability (Sonnenberg and Liem, 2007). In particular, loss of the neuronal

cytoskeletal linking protein dystonin has been attributed to degeneration of both sensory and motor neurons (Brown *et al.*, 1995; Guo *et al.*, 1995; De Repentigny *et al.*, 2011).

Dystonia musculorum (*dt*) is an inherited homozygous recessive sensory neuropathy caused by mutations in the dystonin gene (*Dst*; Duchen *et al.*, 1964). At approximately 2 wk postnatal development, *dt* mice exhibit loss of control of the fore limbs, hind limbs, and trunk, and they die shortly thereafter of unknown causes (Duchen, 1976). Several *dt* alleles exist through spontaneous mutations (*dt*<sup>27</sup>), chemically induced mutations, targeted alleles (*dt*<sup>tm1Efu</sup>), and transgenic insertions (*dt*<sup>Tg4</sup>; Pool *et al.*, 2005). Although only three *dt* mutations (*dt*<sup>Tg4</sup>, *dt*<sup>tm1Efu</sup>, and *dt*<sup>Alb</sup>) have been characterized at the DNA level, *dt*<sup>Tg4</sup> and *dt*<sup>27</sup> are allelic and do not complement (Kothary *et al.*, 1988; Guo *et al.*, 1995). To date, *dt* pathologies have been recorded in motor neurons, skeletal muscle, and Schwann cells, but degeneration is most prominent in the sensory neurons of the dorsal root ganglia (DRG; Dowling *et al.*, 1997; Bernier *et al.*, 1998; Dalpe *et al.*, 1999; De Repentigny *et al.*, 2011).

The *Dst* gene is exceptionally large (~400 kb in mice) and gives rise to three tissue-specific dystonin isoforms—dystonin-e (epithelial isoform, ~315 kDa), dystonin-b (muscle isoform, ~834 kDa), and

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Abbreviations used: DMC, dynein motor complex; DRG, dorsal root ganglia; DRGN, DRG sensory neuron; *Dst*, dystonin gene; *dt*, dystonia musculorum; MACF1b, microtubule actin cross-linking factor-1b; TdT, terminal deoxynucleotidyl transferase; UPR, unfolded protein response.

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dystonin-a (neuronal isoform, ~615 kDa; Sawamura *et al.*, 1991; Brown *et al.*, 1995; Leung *et al.*, 2001; Okumura *et al.*, 2002). Whereas dystonin-e serves as an autoantigen in the skin blistering disease bullous pemphigoid, loss of function of the dystonin-a isoform or isoforms is causal in the *dt* disorder (Kothary *et al.*, 1988; Sawamura *et al.*, 1991; Brown *et al.*, 1995; Pool *et al.*, 2005). Like muscle, three major neuronal isoforms are derived through alternative splicing, namely dystonin-a1, dystonin-a2, and dystonin-a3 (Young and Kothary, 2007). These isoforms share an N-terminal actin-binding domain, an extensive coiled-coil region, and a C-terminal microtubule-binding domain, allowing for interactions with cytoskeletal filaments (Leung *et al.*, 2001). Although the dystonin-a isoforms share similar domain architecture, it is the unique N-terminal regions that differentiate them and dictate their subcellular localization. Specifically, dystonin-a1 encodes a short N-terminal domain that includes an actin-binding domain, whereas dystonin-a2 possesses a transmembrane domain and dystonin-a3 possesses a putative myristoylation domain, aiding their membrane localization to the nuclear envelope and perinuclear membranes and to the plasma membrane, respectively (Jefferson *et al.*, 2006; Young *et al.*, 2006). Recently it has been demonstrated that the lack of one copy of the *Dst* gene or the presence of truncated forms of the mutant protein in humans can lead to a partial disorganization of the sensory and motor circuits. Giorda and collaborators described a human subject with a translocation in the *Dst* gene that specifically disrupted neuron- and muscle-specific isoforms and was associated with a profound delay in the acquisition of cognitive and motor skills, as well as in visual maturation (Giorda *et al.*, 2004). Thus, although no mutations in *Dst* have been identified in humans, haploinsufficiency or altered expression can result in phenotypic abnormalities reminiscent of the inherited disorder in mice.

A common cellular pathology preceding *dt* sensory neuron degeneration is axonal swelling, characterized by the accumulation of organelles and vacuoles within *dt* sensory neurons (Janota, 1972). These accumulations are accompanied by disorganized neurofilament, actin and microtubule networks in *dt* sensory neurons (Yang *et al.*, 1996, 1999; Leung *et al.*, 1999a), which in turn lead to bidirectional impairment of fast axonal transport (Dalpe *et al.*, 1998; De Repentigny *et al.*, 2003). Although neurofilament defects do exist in *dt* mice, they are not causal in initiation of *dt* pathology (Bernier and Kothary, 1998; Eyer *et al.*, 1998). Moreover, the microtubule cytoskeleton of *dt* and wild-type (WT) sensory neurons grown in culture displays no ultrastructural defects, and local trafficking of mitochondrial organelles proceeds normally (Pool *et al.*, 2006). Nonetheless, enhanced apoptosis is observed in these cultures (Pool *et al.*, 2006). Consequently, it is plausible that abnormalities aside from axonal cytoskeleton defects may initiate or contribute to *dt* sensory neuron pathology. Some studies suggest that the dystonin-a2 isoform is involved in nuclear envelope structuring, nuclear tethering, and organization of membranous structures surrounding the nucleus (Young *et al.*, 2003, 2006; Young and Kothary, 2008). Indeed, *dt<sup>Tg4</sup>* sensory neurons (devoid of dystonin-a1 and dystonin-a2 expression) display perikaryal defects and show altered expression of the microsomal enzyme protein disulfide isomerase (Young and Kothary, 2008). Yet the mechanism resulting in perikaryal defects in *dt* remains uncertain. Taken together, the observations make it reasonable to presume that dystonin-a has a role in maintaining integrity of the ER and nuclear envelope, and subsequently organelle function, but this hypothesis has yet to be empirically validated.

Whereas *dt* pathology has been thoroughly examined, there remains a dearth of evidence to explain the mechanisms mediating *dt*

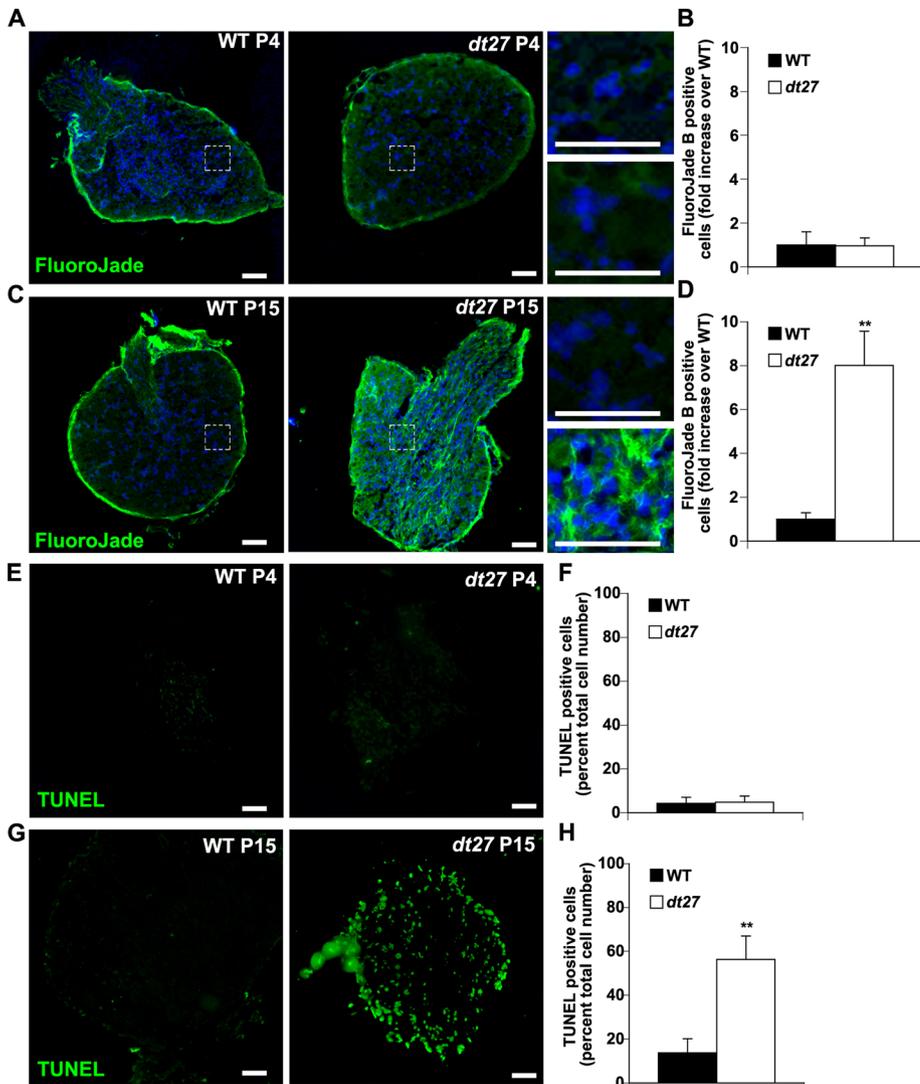
pathogenesis. Moreover, although it is known that the *dt<sup>Tg4</sup>* mouse strain is devoid of dystonin-a1 and dystonin-a2 expression, it is unresolved as to whether loss of a single isoform or combination of isoforms is responsible for neurodegeneration. The present study therefore aims to elucidate underlying mechanisms of *dt* pathogenesis and to determine the individual roles of dystonin-a1 and dystonin-a2 in *dt* sensory neuronal degeneration. To this extent, we selectively reduced the expression of dystonin-a1 or dystonin-a2 via small interfering RNA (siRNA) in F11 neuronal cells and screened these cells for canonical endoplasmic reticulum (ER) stress proteins and caspase activity and assessed cell viability using various cell death assays. In parallel, *dt<sup>27</sup>* DRGs and primary sensory neurons were screened for ER stress, caspase activation, and sensory neuron viability using similar experimental analysis. To assess *dt* ER integrity and function, ultrastructural and ratiometric Ca<sup>2+</sup> analyses were conducted, respectively. Data presented here provide insight into the mechanisms underlying neuropathology in *dt* mice. Furthermore, this study proposes a novel role for dystonin-a2 as a critical structural component of perinuclear organelles, required for maintenance of ER homeostasis.

## RESULTS

### Loss of dystonin-a2 leads to activation of a neurodegenerative caspase cascade in dystonia musculorum mice

To evaluate the isoform-specific contribution of dystonin loss to neurodegeneration in *dt* mice, we first evaluated the correlation between degeneration of sensory neurons and phenotypic onset in *dt<sup>27</sup>* mice, which are null for all three neuronal isoforms of dystonin. At the prephenotypic stage (P4), DRGs from WT and *dt<sup>27</sup>* mice were negative for both Fluoro-Jade B staining and terminal transferase dUTP nick-end labeling (TUNEL; Figure 1, A, B, E, and F), indicating the absence of neurodegeneration. In phenotypic (P15) *dt<sup>27</sup>* animals, however, a marked increase in both Fluoro-Jade staining and TUNEL labeling (Figure 1, C, D, G, and H) was observed relative to WT littermates. This result indicates that phenotypic onset correlates closely with severe degeneration of DRG sensory neurons. We then sought to determine which neuronal isoform of dystonin was critical to maintaining cell viability in sensory neurons. *dt<sup>Tg4</sup>* transgenic mice are null for dystonin-a1 and dystonin-a2 but express dystonin-a3 (Pool *et al.*, 2005). Labeling of P15 WT and *dt<sup>Tg4</sup>* DRGs for TUNEL shows an increase in TUNEL reactivity in *dt<sup>Tg4</sup>* DRGs relative to WT (Supplemental Figure S1). This indicates that the neurodegeneration associated with dystonin loss in *dt<sup>Tg4</sup>* mice is not due to loss of dystonin-a3 but rather to loss of dystonin-a1 or dystonin-a2 or the combined loss of both.

The diverging cellular localization of dystonin-a1 and dystonin-a2 resulting from the heterogeneity of the N-terminus would implicate distinct organelle-specific dysfunction with their depletion (Young and Kothary, 2008). To determine the functional requirement of both dystonin-a1 and dystonin-a2 to neuronal viability, we performed isoform-specific loss-of-function analysis in immortalized F11 neuronal culture. F11 neuronal cells are a fusion of embryonic rat DRG neurons with mouse neuroblastoma cells. The resulting hybrid exhibits neuronal gangliosides, action potentials, and extensive neurite-like processes (Platika *et al.*, 1985). We first assessed the efficiency of isoform-specific, siRNA-mediated dystonin knockdown. In the absence of isoform-specific dystonin antibodies, we assessed knockdown of exogenously expressed dystonin-a1–green fluorescent protein (GFP) and dystonin-a2–yellow fluorescent protein (YFP) fusion proteins coupled to siRNA-mediated knockdown of endogenously expressed dystonin-a1 and dystonin-a2 mRNA (Figure 2, A



**FIGURE 1:** Dystonin deficiency leads to degeneration of sensory neurons in mice. DRGs from P4 (A, B) and P15 (C, D) WT and *dt<sup>27</sup>* mice were stained with Fluoro-Jade B to identify degenerating neurons. No degeneration of sensory neurons is evident prephenotype (A, B), whereas Fluoro-Jade–positive degenerating neurons were evident at P15 in phenotypic *dt<sup>27</sup>* animals (C, D). Analysis of TUNEL labeling from P4 (E, F) and P15 (G, H) WT and *dt<sup>27</sup>* DRGs yielded no difference in apoptotic index prephenotype (E, F). At P15, a significant increase in percentage TUNEL-positive cells was observed in phenotypic *dt<sup>27</sup>* animals relative to WT controls (G, H). Scale bars, 50  $\mu$ m; Student's *t* test, \*\**p* < 0.01, *n* = 5.

and B, and Supplemental Figure S2, A and B). siRNA-mediated targeting of dystonin proved to be isoform specific. Analysis of the effect of dystonin loss on neuronal viability showed that knockdown of dystonin-a2 resulted in an increase in death within 24 h as indicated by ethidium homodimer incorporation (Figure 2C and Supplemental Figure S2C) and an increase in TUNEL labeling within 48 h (Figure 2D) of silencing relative to scrambled control. Loss of dystonin-a1 has no impact on cell death (Figure 2, C and D, and Supplemental Figure S2C). Positive TUNEL labeling in phenotypic DRGs from *dt<sup>27</sup>* mice coupled with TUNEL labeling following specific depletion of dystonin-a2 in F11 neuronal cells led us to explore the pathway of apoptotic induction in order to trace to the mechanism of pathology.

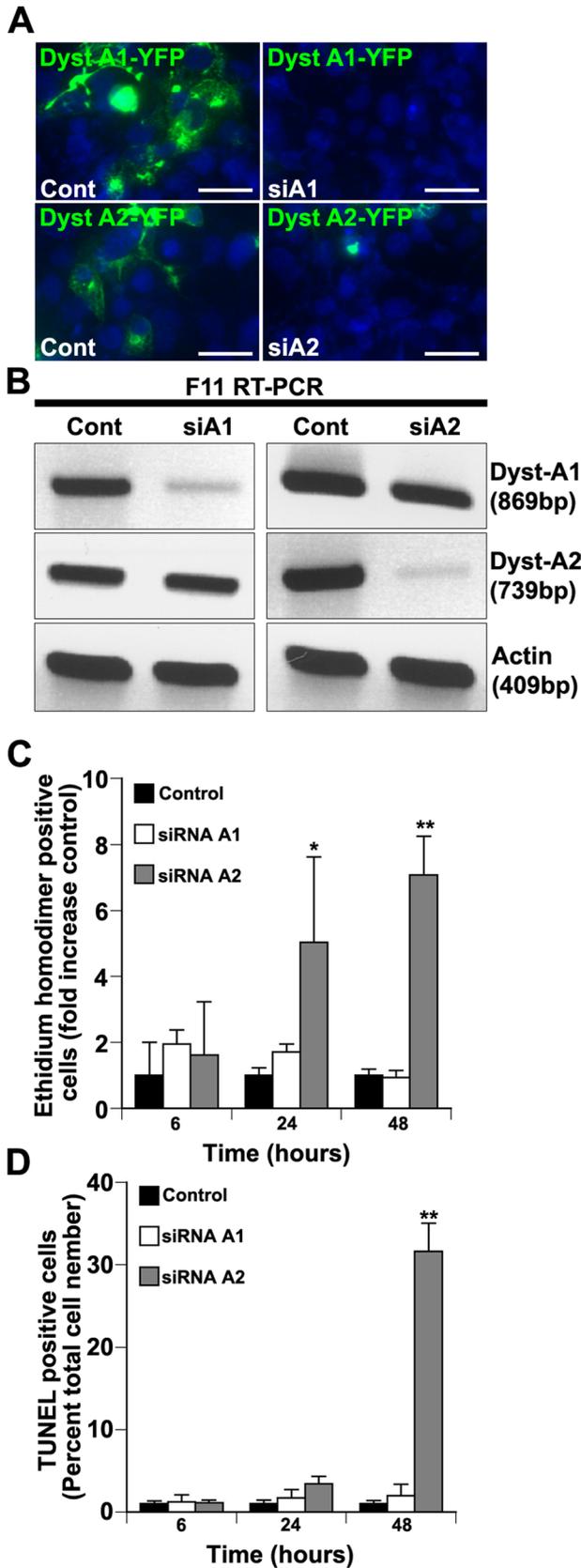
We thus screened for caspase activation in F11 cells following siRNA-mediated depletion of dystonin isoforms. Using fluorescence-labeled inhibitors of caspase activity, we screened F11 neuronal

cells for activation of caspases 2, 12, 8, and 3 at multiple time points following loss of either dystonin-a1 or dystonin-a2 (Figure 3). No induction of death receptor–associated caspase 8 was detected (Figure 3A), thus excluding the extrinsic apoptotic cascade as a potential mediator of death. A pronounced increase in both caspase 2 and caspase 3 activity was observed (Figure 3, C and D) following 24 and 48 h of dystonin-a2 depletion relative to scrambled siRNA control cells, coupled to a modest increase in caspase 12 activation (Figure 3B) following 48 h of dystonin-a2 depletion. Loss of dystonin-a1, however, had no effect on caspase activation. Collectively, these data strongly suggest that specific depletion of dystonin-a2 induces an intrinsic apoptotic cascade that is a likely effector of neurodegeneration in *dt*.

### Loss of dystonin results in ER stress–mediated induction of the unfolded protein response

To ascertain the cellular stresses evoking the increase in caspase activity in F11 neuronal cells, we first discerned which if any of these caspases contributed to pathology in *dt<sup>27</sup>* mice by screening WT and *dt<sup>27</sup>* primary sensory neurons for caspase activation. A time course of caspase 3 activation was conducted in DRG sections at P4, P10, and P15 (Figure 4A). These data suggest that caspase 3 activation in DRGs coincides with phenotypic onset in *dt<sup>27</sup>* mice. We then established the ability of these neurons to survive in culture. Cultured DRG sensory neurons (DRGNs) from P4 and P15 WT and *dt<sup>27</sup>* mice were assessed by TUNEL (Figure 4B). Whereas both WT and *dt<sup>27</sup>* cultures were negative for TUNEL labeling at P4 (Figure 4C), we were surprised to see that P15 DRGNs from *dt<sup>27</sup>* mice also showed no increase in TUNEL labeling relative to WT controls (Figure 4D). We then screened P15 WT and *dt<sup>27</sup>* sensory neurons for caspase activation (Figure 4E). Although we saw no induction of death receptor–associated caspase 8 and no significant increase in ER-associated caspase 12 activity, an increase in activity of executioner caspases 2 and 3 was observed. Activation of caspase 2 in whole DRGs was confirmed by Western analysis of cleaved (active) caspase 2 at P4 (Figure 4, F and G) and P15 (Figure 4, F and H). Caspase activation was only observed in DRGs from phenotypic *dt<sup>27</sup>* mice (Figure 4, F and H). In addition, although these results suggest that culturing sensory neurons at a phenotypic stage selects for those neurons that have not yet undergone apoptosis, a caspase 2–dependent signaling cascade has nonetheless been initiated.

In rodents, ER stress leading to neuronal loss is mediated in part by activation of caspase 12 (Nakagawa et al., 2000). Caspase 2, however, has been proposed as a functional orthologue to caspase 12 in some systems, mobilized in response to ER stress to activate downstream caspase 3/7 (Hitomi et al., 2004; Cheung

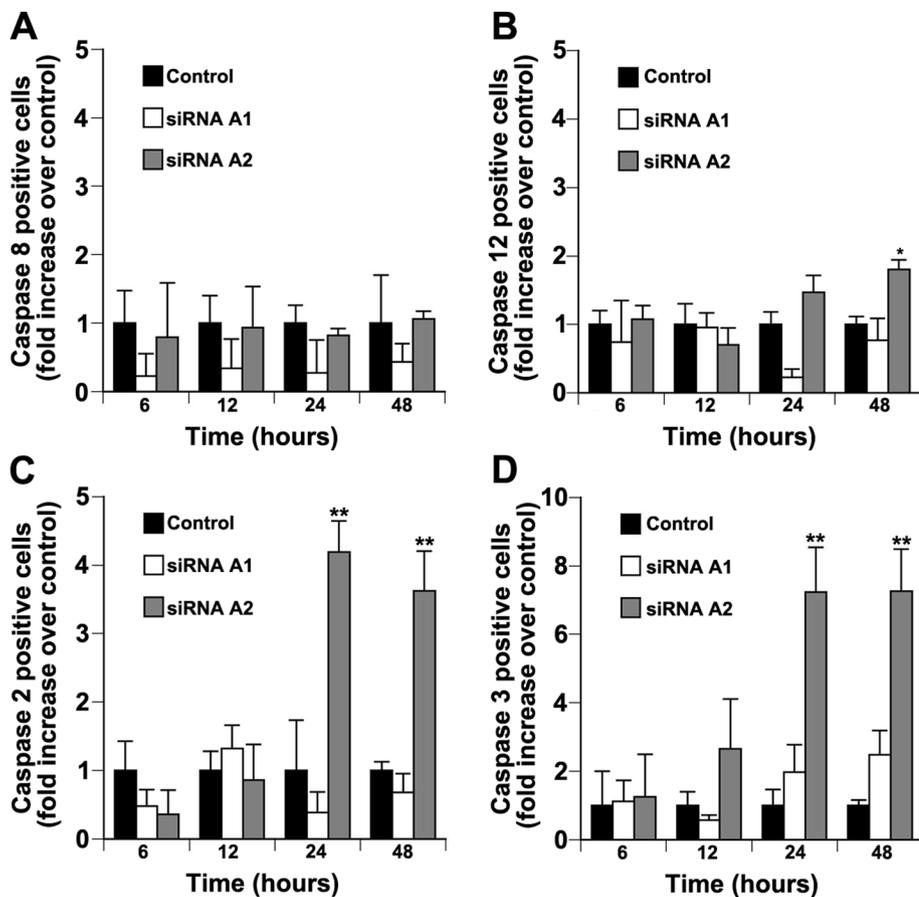


**FIGURE 2:** Isoform-specific loss of dystonin-a2 promotes neuronal cell death. (A) Efficiency of siRNA-mediated knockdown of exogenously expressed dyst-a1-YFP and dyst-a2-YFP protein was evaluated in COS-1 cells by epifluorescence. (B) Efficiency of siRNA-mediated knockdown of endogenously expressed dystonin-a1

et al., 2006). Caspase 2 localizes to both the Golgi apparatus and ER (Mancini et al., 2000; Ferri and Kroemer, 2001; Cheung et al., 2006). Activation is associated with fatal interruption of protein transport from the ER to the Golgi, triggering cell death (Murakami et al., 2007; Ouasti et al., 2007). To evaluate whether caspase 2 was localized to the ER and functioning as a component of ER stress following dystonin loss, we contrasted caspase 2 localization in F11 neuronal cells between the ER and Golgi and determined whether dystonin-a2 depletion altered this localization (Figure 5). Confocal analysis of caspase 2 with the ER marker calreticulin-YFP showed extensive colocalization (Figure 5A). Furthermore, loss of dystonin-a2 has no effect on the distribution of caspase 2 in the ER (Figure 5, B and E). Assessment of caspase 2 colocalization with Golgi-YFP also showed caspase 2 present in Golgi (Figure 5C). Consistent with our findings in the ER, loss of dystonin-a2 has no effect on Golgi-localized caspase 2 (Figure 5, D and F). Although dystonin-a2 has no effect on overall caspase 2 distribution, we see extensive caspase 2 staining through both the ER and Golgi complex, suggesting that caspase 2 is positioned in F11 neuronal cells to respond to ER stress signals. We thus examined the effect of dystonin-a2 depletion on the macrostructural organization of the ER. It was surprising that staining of endogenous calreticulin in F11 neuronal cells showed no obvious aberrations in ER macrostructure (Figure 5G).

Further elucidation of the ER stress mechanism associated with neurodegeneration in *dt<sup>27</sup>* mice focused on two canonical ER stress signal transduction cascades—mobilization of ER Ca<sup>2+</sup> stores and the unfolded protein response (UPR). Phenotypic WT and *dt<sup>27</sup>* DRGs were analyzed by Western blot for induction of chaperones of protein folding—BiP (GRP78) and CHOP (GADD153). Induction of these proteins is an early marker of ER stress (Gulow et al., 2002). Expression of these proteins increases following cessation of translation induced by UPR activation (Munro and Pelham, 1986; Gulow et al., 2002). A significant induction of both BiP and CHOP was observed in DRGs from phenotypic *dt<sup>27</sup>* mice relative to WT (Figure 6, A–C). Isoform-specific depletion of dystonin was subsequently performed to ascertain which dystonin isoform is critical to ER function. Western and immunofluorescence analysis of F11 neuronal cells revealed an increase in BiP and CHOP expression within 48 h of dystonin-a2 silencing but not following dystonin-a1 silencing (Figure 6, D–I). UPR signal transduction results in the mRNA splicing of XBP1 by IRE1 (Yoshida et al., 2001). Translation of spliced XBP1 mRNA, a potent transcription factor, induces BiP and CHOP expression (Lee et al., 2003). UPR-mediated activation of XBP1 splicing was determined following isoform-specific depletion of dystonin. Although dystonin-a1 depletion resulted in a modest induction of XBP1 splicing, a dramatic increase in splicing was observed following loss of dystonin-a2 (Figure 6, J and K). Collectively, these data demonstrate that dystonin-a2 functional loss is the predominant mediator of neurodegeneration in *dt<sup>27</sup>* mice through induction of an ER stress-mediated apoptotic cascade.

and dystonin-a2 mRNA was measured in F11 neuronal cells by RT-PCR. Knockdown of dystonin-a2 resulted in an increase in death as assessed by ethidium homodimer incorporation within 24 h (C) and an increase in TUNEL labeling within 48 h of silencing relative to scrambled control. Loss of dystonin-a1 has no impact on cell death (C, D). Scale bars, 10 μm; ANOVA, post hoc Dunnett's t test, \*p < 0.05, \*\*p < 0.01, n = 5.



**FIGURE 3:** Loss of neuronal dystonin-a2 results in activation of a caspase 2–dependent caspase cascade in F11 cells. F11 neuronal cells were screened for caspase activation using FLICA assays at multiple time points following knockdown of either dystonin-a1 or dystonin-a2. (A) No induction of death receptor–associated caspase 8 was observed at any time point. (B) A modest increase in caspase 12 activation was measured following 48 h of dystonin-a2 depletion. A pronounced increase in both caspase 2 (C) and caspase 3 (D) activity was observed following 24 and 48 h of dystonin-a2 depletion relative to scrambled siRNA control cells. Loss of dystonin-a1 had no effect on caspase activation (A–D). ANOVA, post hoc Dunnett’s *t* test, \**p* < 0.05, \*\**p* < 0.01, *n* = 5.

### ER structural integrity and calcium homeostasis are perturbed in *dt<sup>27</sup>* mice

We previously reported that the N-terminal transmembrane domain of dystonin-a2 localizes the protein to the ER and that overexpression of dystonin-a2 results in reorganization of ER membranes (Young and Kothary, 2008). We thus postulated that ER stress induced by loss of the dystonin-a2 isoform might manifest as a result of perturbed cytoarchitecture within the ER and/or the impaired anchoring of ER membrane to the cytoskeleton. To test these possibilities further, we performed a time course of electron microscopy on WT and *dt<sup>27</sup>* DRGs and evaluated ER morphology within sensory neurons (Figure 7, A–C). Normal striated patterning of ER can be identified in WT and *dt<sup>27</sup>* DRGs at P4; however, by P10 the ER of *dt<sup>27</sup>* DRGs appear dilated. This pathology increases in severity by P15, and in some instances the ER appears vacuolated, suggesting impaired ER cytoskeletal dynamics (Figure 7D). This ER pathology precedes phenotypic onset in *dt<sup>27</sup>* mice.

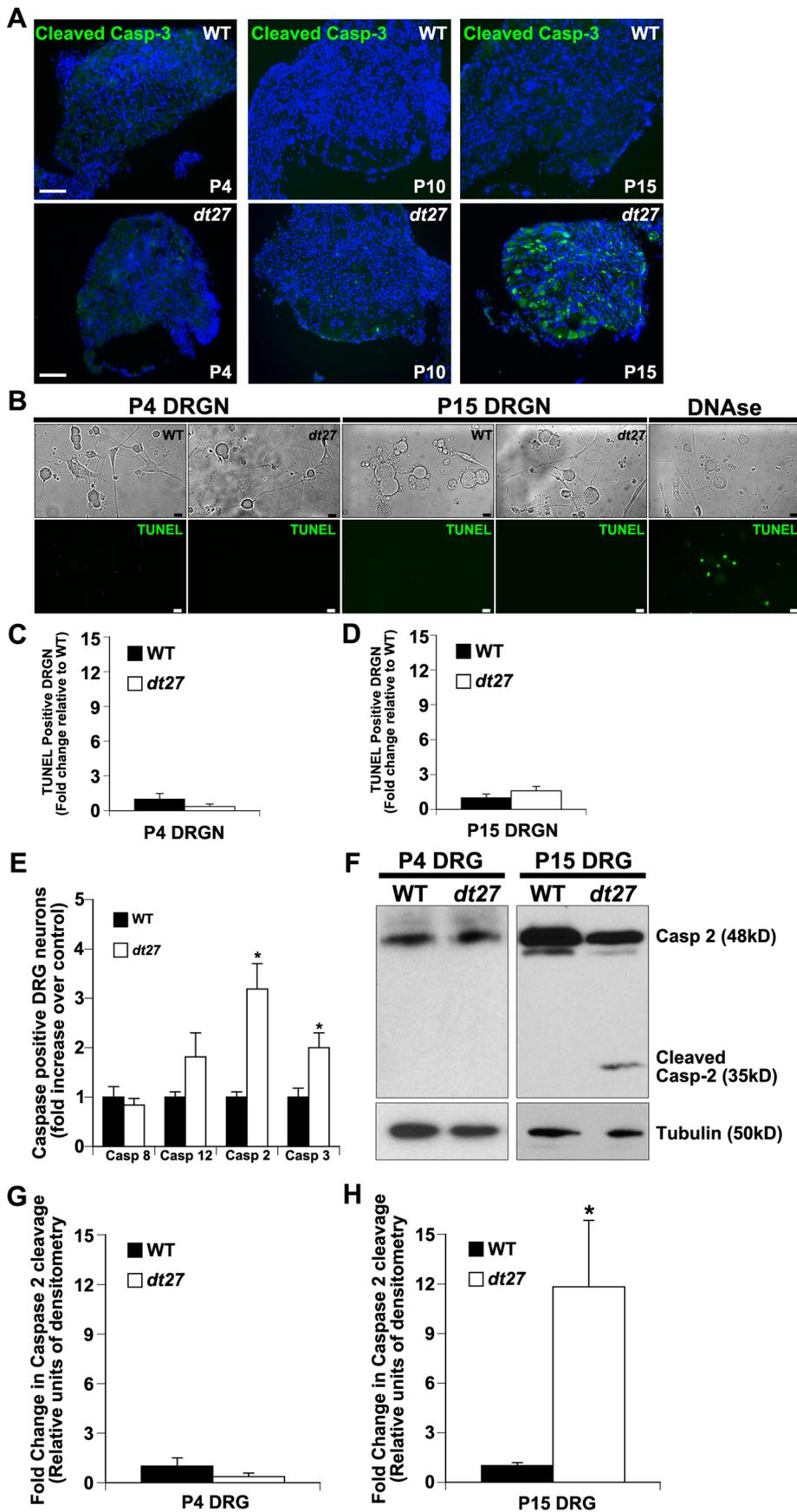
We then evaluated whether a defect in ER morphology correlates with aberrant Ca<sup>2+</sup> homeostasis, thus initiating ER stress signal transduction. WT and *dt<sup>27</sup>* primary sensory neurons were visualized and recorded using the Ca<sup>2+</sup>-sensitive dye Fura-2AM. Neurons were depleted of Ca<sup>2+</sup> by perfusion in Ca<sup>2+</sup>-free tyrode

solution. Cells were then stimulated with caffeine to promote ryanodine receptor-mediated Ca<sup>2+</sup> efflux from the ER (Ehrlich *et al.*, 1994). *dt<sup>27</sup>* sensory neurons showed reduced ER calcium efflux relative to WT sensory neurons as indicated by a reduction in maximum peak amplitude following caffeine administration (Figure 7, E–G). Ca<sup>2+</sup> reuptake was normal in both WT and *dt<sup>27</sup>* sensory neurons as indicated by the second peak response following caffeine administration. To confirm that the observed peak response was indeed a result of Ca<sup>2+</sup> efflux from the ER, following caffeine administration, neurons were perfused for 3 min with thapsigargin to block Ca<sup>2+</sup> reuptake by sarco/endoplasmic reticulum Ca<sup>2+</sup>-ATPase (SERCA) pumps (Lytton *et al.*, 1991). Following SERCA inhibition, caffeine stimulation did not result in Ca<sup>2+</sup> mobilization, confirming that caffeine was indeed stimulating efflux from ER stores alone and that all Ca<sup>2+</sup> in the ER was effluxed following stimulation with caffeine (Figure 7, H and I). We then further evaluated which dystonin isoform was primarily responsible for maintaining ER Ca<sup>2+</sup> homeostasis. Mobilization of ER Ca<sup>2+</sup> stores was measured following isoform-specific dystonin silencing in F11 neuronal cells (Supplemental Figure S3). Isoform-specific loss of dystonin-a2 resulted in a decrease in maximum peak amplitude relative to control siRNA-treated cells, whereas dystonin-a1 depletion had no effect on ER Ca<sup>2+</sup> efflux (Supplemental Figure S3, A–D). Taken together, these data suggest that impaired cytoarchitecture at the ER results in ER dilation and a reduction in the steady-state levels of ER Ca<sup>2+</sup>. This in turn would

promote the observed ER stress-mediated apoptotic signaling cascade.

### Inhibition of ER stress signal transduction rescues dystonin-deficient neurons from death

Finally, to confirm that ER stress was the primary source of neuronal degeneration in *dt<sup>27</sup>* mice, we attempted to rescue neurons from cell death by intervening in the apoptotic signal cascade identified (Figure 8). We first assessed whether various caspase inhibitors could prevent downstream executioner caspase 3 activation in F11 neuronal cells following loss of dystonin-a2. Pan-caspase inhibition or inhibition of caspase 2 but not caspase 12 prevented downstream caspase 3 activation in F11 neuronal cells (Figure 8A). This was confirmed in primary sensory neurons from *dt<sup>27</sup>* mice, in which pan-caspase inhibition in addition to inhibition of either caspase 2 or caspase 12 prevented downstream caspase 3 activation (Figure 8B). These data confirmed that ER-associated caspase activation is critical to neurodegeneration in *dt<sup>27</sup>*. We then sought to block UPR signal transduction directly and evaluate the effect on cell viability. Salubrinal is a selective inhibitor of cellular complexes that dephosphorylate eIF2 $\alpha$ , a downstream effector of UPR-mediated protein kinase R–like ER kinase (PERK) activation (Boyce *et al.*, 2005).



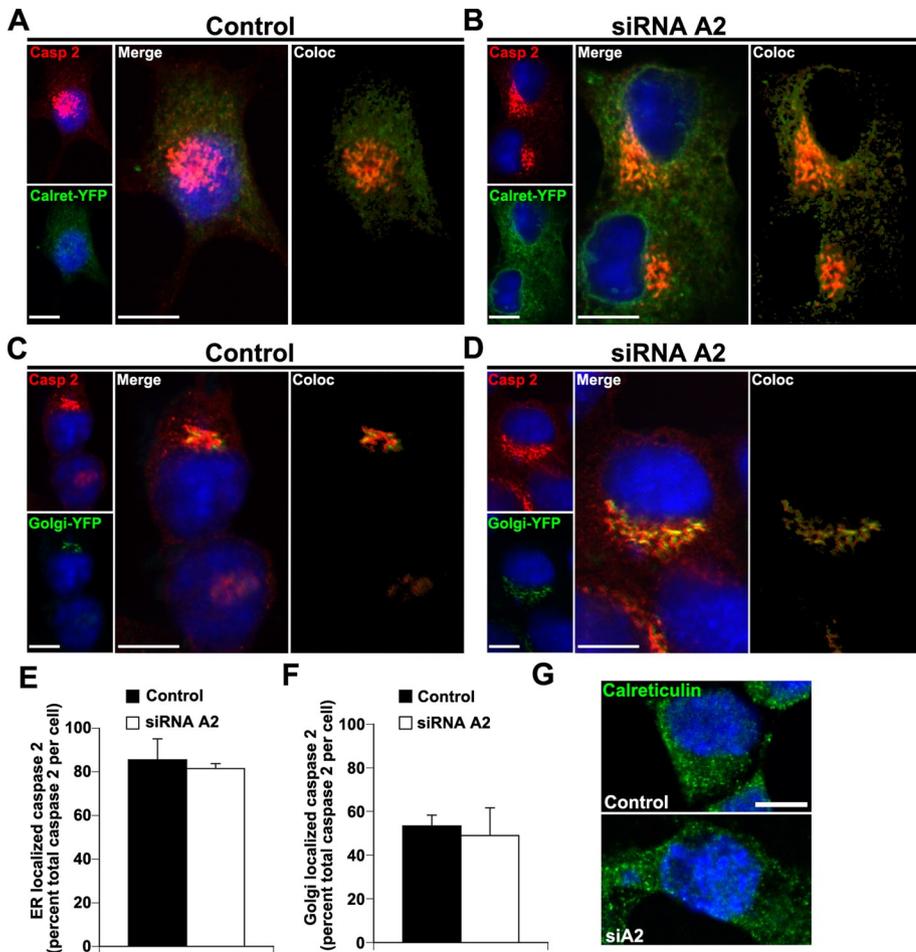
**FIGURE 4:** Loss of dystonin results in activation of an ER-associated caspase cascade in DRGs. (A) A postnatal time course of caspase 3 activity was conducted by immunofluorescence analysis of cleaved caspase 3 expression at P4, P10, and P15 in WT and *dt<sup>27</sup>* DRG sections; scale bars,

Western analysis of F11 neuronal cells treated with various concentrations of salubrinal confirmed that it inhibits downstream UPR signaling by preventing dephosphorylation of eIF2 $\alpha$  (Figure 8C). Fold change in ethidium homodimer incorporation showed that whereas dystonin-a2 depletion causes a significant increase in death, salubrinal treatment significantly reduces this death (Figure 8, D and E). Finally, we evaluated the effect of salubrinal treatment on viability of *dt<sup>27</sup>* primary sensory neurons as assessed by ethidium homodimer incorporation. Here we show that UPR blockade with 25  $\mu$ M salubrinal prevents degeneration in primary *dt<sup>27</sup>* sensory neurons. In summary, these data highlight a critical role for dystonin-a2 in mediating normal ER structure and function. Loss of this protein leads to the ER stress and apoptosis that underlie the sensory neurodegeneration observed in *dt<sup>27</sup>* mice.

## DISCUSSION

Mutations in the *Dst* gene that encodes all isoforms of the giant cytoskeletal cross-linking protein dystonin result in sensory neurodegeneration in *dt* mice (Bernier *et al.*, 1995). The consensus regarding this degenerative event is that it arises due to loss of structural organization of cytoskeletal elements (Yang *et al.*, 1996, 1999; Leung *et al.*, 1999a). The numerous functions of the cytoskeleton have made elucidation of the mechanism underlying *dt* pathogenesis challenging. Here we identify a signaling cascade resulting from dystonin deficiency in sensory neurons by which loss of organelle integrity that

50  $\mu$ m. (B) Survival of cultured primary sensory neurons was then assessed by TUNEL labeling of WT and *dt<sup>27</sup>* DRGNs at P4 and P15; scale bars, 10  $\mu$ m. DNase-treated sensory neurons were processed as a positive control. Fold change in TUNEL reactivity was quantified at (C) P4 and (D) P15. Data are expressed as fold increase in TUNEL-positive *dt<sup>27</sup>* neurons relative to WT; Student's *t* test, *n* = 3–6. (E) WT and *dt<sup>27</sup>* primary sensory neurons were screened for caspase activation following 48 h in culture using FLICA assays. Although no induction of death receptor-associated caspase 8 was measured, an increase in activity of executioner caspases 2 and 3 was recorded; ANOVA, post hoc Dunnett's *t* test, \**p* < 0.05, *n* = 9. (F) Caspase 2 activity was confirmed by Western analysis of pro-caspase 2 cleavage in both P4 and P15 WT and *dt<sup>27</sup>* DRGs. Caspase 2 cleavage in (G) P4 and (H) P15 WT and *dt<sup>27</sup>* DRGs was quantified by densitometry and normalized to tubulin standard; Student's *t* test, \**p* < 0.05, *n* = 5.



**FIGURE 5:** Caspase 2 is ER associated in F11 neuronal cells. F11 cells overexpressing either calreticulin-YFP (A, B) or Golgi-YFP (C, D) were antigenically labeled for caspase 2 following 48-h treatment with either (A, C) control or (B, D) dystonin-a2 siRNAs (scale bars, 10  $\mu$ m). Colocalization of caspase 2 with either the ER (calreticulin) or Golgi was subsequently determined and colocalization masks generated. Quantification of the percentage colocalized area between caspase 2 and (E) the ER or caspase 2 and (F) the Golgi was subsequently performed; Student's *t* test, *n* = 12. Loss of dystonin has no effect on localization. (G) ER macrostructure was visualized by antigenic labeling of endogenous calreticulin (scale bars, 10  $\mu$ m). Loss of dystonin-a2 had little effect on calreticulin subcellular organization.

precedes phenotypic onset in *dt*<sup>27</sup> mice triggers ER-mediated apoptosis (Figure 9). We observe ultrastructural dilation of the ER that correlates with Ca<sup>2+</sup> dyshomeostasis and activation of canonical ER stress proteins. This in turn leads to activation of ER stress caspases 2 and 12, culminating in apoptosis. Furthermore, we propose these pathologies to be the consequence of specific loss of dystonin-a2.

### Dystonin as a regulator of ER stress

The ER is involved in transduction of apoptotic signaling through two pathways—UPR and Ca<sup>2+</sup> mobilization. UPR is initiated to restore protein folding in the ER and can itself lead to ER dilation through autophagy (Hoyer-Hansen and Jaattela, 2007). Apoptosis is initiated when ER function cannot be restored (Hoyer-Hansen and Jaattela, 2007). Ca<sup>2+</sup> mobilization from the ER results in activation of the cysteine protease *m*-calpain (Orrenius et al., 2003). Calpain activation promotes apoptosis through activation of ER-associated caspase 12 (Nakagawa and Yuan, 2000). Alternatively, activation of caspase 2 depends on both cleavage and phosphorylation by Ca<sup>2+</sup>-dependent CaMKII (Nutt et al., 2005). Caspase 2 propagates apoptosis through activation of executioner caspases and cleavage

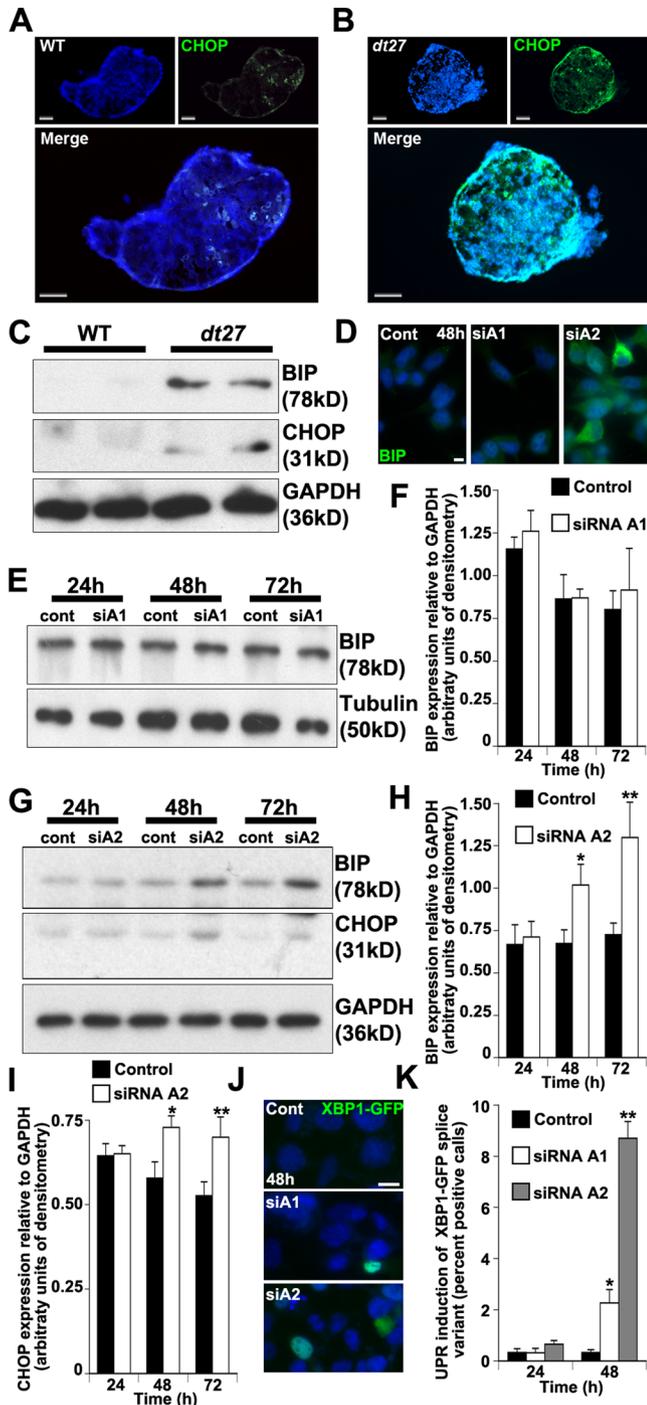
of cytoskeletal linker protein  $\alpha$ II-spectrin. In *dt*, we find a pronounced neuroprotection effected by caspase blockade and a partial rescue of neuronal viability by the UPR inhibitor salubrinal. The dependence of caspase 2 and 12 activation on Ca<sup>2+</sup> signal transduction implicates ER-Ca<sup>2+</sup> dyshomeostasis as a primary inducer of neurodegeneration in *dt*.

Molecular interaction studies implicated dystonin in regulation of the dynein motor complex (DMC) through interaction with dy-nactin and the endosomal vesicle protein retrolinkin (Liu et al., 2003, 2007). It is intriguing to speculate how this interaction may implicate cytoskeletal linker proteins as master regulators of ER function. Mutations in the cytoskeletal linker protein  $\beta$ III-spectrin cause severe cerebrosplinal ataxia in murine and *Drosophila* models (Lorenzo et al., 2010; Perkins et al., 2010). The ataxic phenotype is enhanced by both dynein and dynactin loss-of-function mutations, implicating aberrant intracellular transport as a mediator of pathology (Lorenzo et al., 2010). The potential role for dystonin as a DMC component, coupled with our findings of a role for dystonin-a2 as mediator of ER integrity, supports the notion that dystonin may regulate components of the endocytic pathway. Indeed, cytoskeletal linkers may have a general role in mediating axonal transport by regulating ER and endosomal organization. As such, their dysfunction may contribute to numerous neurodegenerative conditions whose etiologies are unknown. Aberrations in the signaling events described previously are associated with neurodegenerative disease. For example, mutations in the ER protein presenilin-1 found in familial Alzheimer disease (Sherrington et al., 1995) result in aberrant A $\beta$  processing (Lemere et al., 1996)

and increased neuronal susceptibility to ER stress (Katayama et al., 1999). ER stress further promotes A $\beta$  generation, propagating disease pathology (Suh and Checler, 2002). A $\beta$  itself mediates ER collapse by interfering with membrane anchoring to the cytoskeleton (Lai et al., 2009).

### Dystonin-a2 and organelle integrity

Initial studies on *dt* mice demonstrated an involvement of the perikaryon of certain sensory neurons in disease. Peripheral displacement of Nissl substance, nuclear eccentricity, and cell enlargement were among defects noted (Duchen et al., 1964; Messer and Strominger, 1980; Sotelo and Guenet, 1988). More recently, investigation of *dt*<sup>Tg4</sup> sensory neurons also documented perikaryal defects, suggesting that abnormalities other than those from cytoskeletal disorganization in the axon could initiate or contribute to *dt* pathogenesis (Young and Kothary, 2008). These initial observations of *dt* perikaryon abnormalities fit well with recent data that demonstrated an N-terminal transmembrane domain contained in dystonin-a2 facilitates its localization to perinuclear membranes (Young et al., 2006). Involvement of



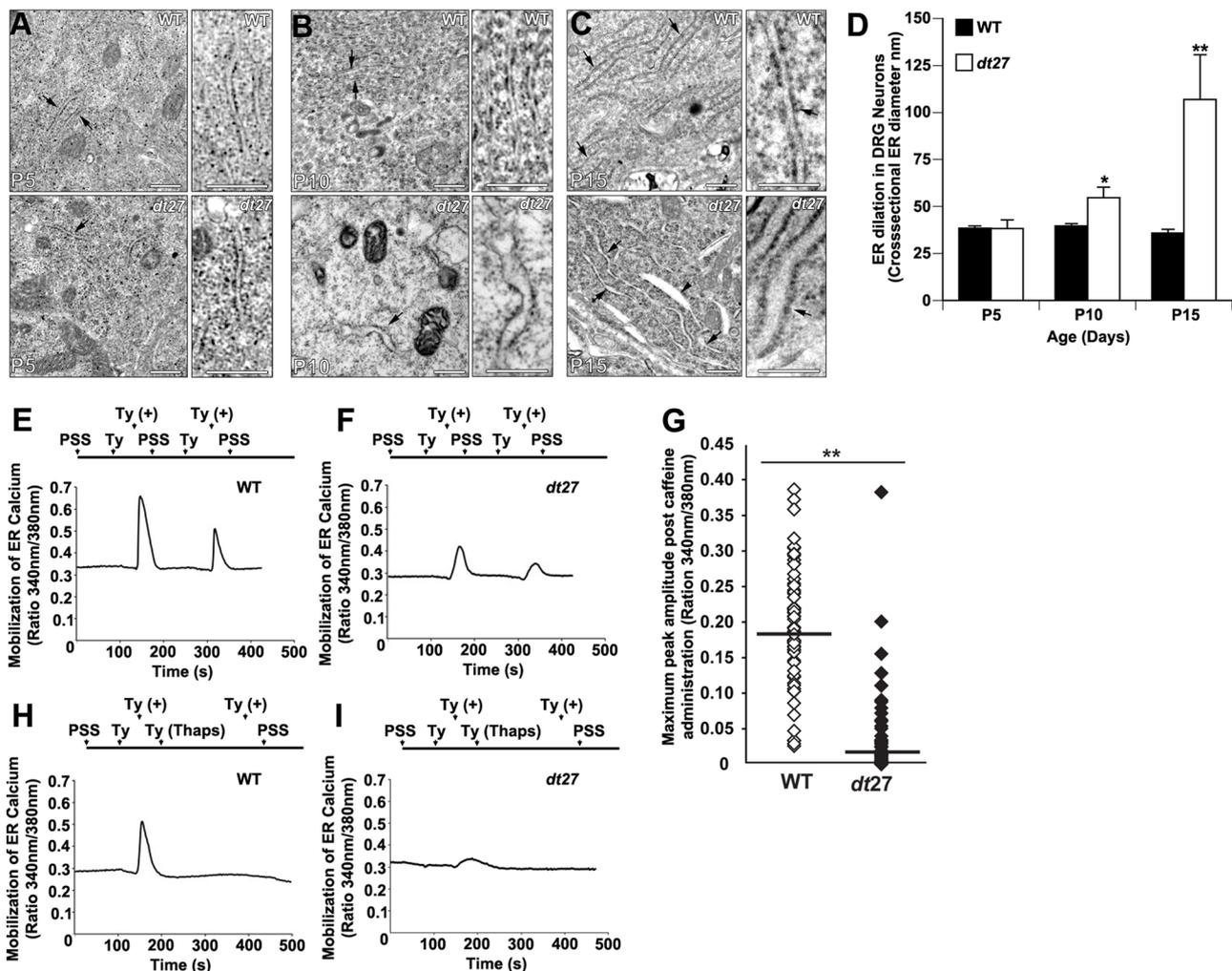
**FIGURE 6:** Loss of dystonin results in ER stress-mediated induction of the unfolded protein response. (A, B) Antigenic labeling of CHOP by immunofluorescence confirmed an induction of CHOP expression in whole DRGs from *dt<sup>27</sup>* animals relative to WT littermate controls (scale bars, 50  $\mu$ m). (C) Phenotypic WT and *dt<sup>27</sup>* DRGs were analyzed by Western blot for induction of UPR proteins BiP and CHOP. A significant induction of both BiP and CHOP was observed in P15 *dt<sup>27</sup>* DRGs (each lane represents pooled DRGs from three animals). (D) Induction of BiP expression following loss of dystonin-a2 was confirmed by immunofluorescence antigenic labeling (scale bars, 10  $\mu$ m). (E–I) Isoform-specific depletion of dystonin was performed in F11 neuronal cells and the effect on UPR induction determined. (E, F) Although Western analysis did not reveal a change in BiP expression following dystonin-a1 silencing, an increase in (G, H) BiP and (G, I) CHOP expression was detected within 48 h of dystonin-a2 silencing;

dystonin-a2 as a structural component of the ER is further supported by the finding that ectopic overexpression of dystonin-a2 fusion protein alters the subcellular localization of calreticulin (Young and Kothary, 2008). Furthermore, altered protein disulfide isomerase expression in *dt<sup>Tg4</sup>* sensory neurons suggested that ER dysfunction or impaired protein folding may come as a result of perturbed ER structure in multiple *dt* mutant alleles (Young and Kothary, 2008).

In the present study, we find ER structural integrity to be perturbed in *dt<sup>27</sup>* sensory neurons (Figure 7C). Although past studies on *dt* sensory neurons highlighted unexplained changes in ER resident proteins, a mechanistic link between aberrant ER ultrastructure, and activation of ER stress signaling cascades has yet to be resolved. ER dilation and vacuole formation can occur as a component of autophagy and is often induced by ER stress as a neuroprotective mechanism to remove damaged organelles (Marino *et al.*, 2011; Scheper *et al.*, 2011). It is surprising that ER dilation in this instance preceded caspase activation (Figure 4) and UPR induction (data not shown). Considering previous related work on the subject, which described dystonin-a2 as an anchor for perinuclear membranes through its attachment of actin microfilaments, we propose compromised organelle structure to result from loss of cytoskeletal interaction. Indeed, it has been established that organization, movement, and proper function of organelles are highly reliant upon the cytoskeleton (Barr and Egerer, 2005; Starr, 2007; Bola and Allan, 2009). An example of organelle integrity being maintained through cytoskeletal linking proteins is found in the microtubule actin cross-linking factor-1b (MACF1b). As a member of the plakin protein family, MACF1b shares similar domain architecture as dystonin-a. It interacts with microfilaments and microtubules and is critical for Golgi complex polarization and stability of the microtubule-organizing complex (Leung *et al.*, 1999b; Kodama *et al.*, 2003; Lin *et al.*, 2005). Although the two proteins are expressed in many of the same tissues, DRG neurons express predominantly dystonin (Leung *et al.*, 2001), which might explain why *dt* degeneration is most prominent in sensory neurons.

Because the functions of organelles are contingent upon organelle structure, the establishment of dystonin-a2 as an ER structural anchor makes it not surprising that  $Ca^{2+}$  dyshomeostasis and UPR activation are observed in *dt* sensory neurons. Hallmark pathologies of many neurodegenerative diseases center on elements of ER dysfunction. Future studies should aim to determine whether abnormal ER morphology causes  $Ca^{2+}$  dyshomeostasis, whether dystonin-a2 interacts with ER-proteins, or whether cytoskeletal stabilization prevents ER stress in other sensory neuropathies. The partial rescue implied by UPR blockade following dystonin-a2 loss indicates that the mechanisms causing death of *dt* sensory neurons are still active. To further discern the functions of dystonin isoforms, we must target upstream of ER defects. Collectively, data presented here provide compelling evidence that dystonin-a2 is a critical mediator of normal ER structure and function.

ANOVA, post hoc Dunnett's *t* test, \**p* < 0.05, \*\**p* < 0.01, *n* = 3–7). (J, K) UPR-mediated activation of XBP1 splicing was then determined by total cell counts of XBP1-GFP expression. A 48-h dystonin-a1 depletion resulted in a modest induction of XBP1 splicing, whereas a dramatic increase in splicing was observed following loss of dystonin-a2 (scale bars, 10  $\mu$ m); ANOVA, post hoc Dunnett's *t* test, \**p* < 0.05, \*\**p* < 0.01, *n* = 3–7).



**FIGURE 7:** ER  $\text{Ca}^{2+}$  homeostasis is perturbed in the sensory neurons of *dt27* mice. Electron microscopy of WT and *dt27* DRGs at (A) P5, (B) P10, and (C) P15. Normal striated patterning of ER can be observed in WT DRGs (A–C top, arrows). In contrast, the ER in *dt27* DRGs is dilated (A–C, bottom arrows), leading to vacuole formation in some instances (C, bottom, arrowhead; scale bars, 500 nm). (D) Quantification of ER dilation shows a significant increase in ER dilation at P10 and P15 in *dt27* DRGs relative to WT; one-way Student's *t* test, \* $p < 0.05$ , \*\* $p < 0.01$ ,  $n = 3$ .  $\text{Ca}^{2+}$  mobilization from the ER of (E) P15 WT and (F) *dt27* primary sensory neurons was visualized and recorded using Fura-2AM dye, and a representative graph is depicted. Neurons were perfused with PSS for 100 s prior to  $\text{Ca}^{2+}$  depletion in tyrode (Ty) buffer for 30 s. Caffeine was then administered for 30 s in tyrode buffer [Ty(+)] to stimulate  $\text{Ca}^{2+}$  efflux from the ER. Following  $\text{Ca}^{2+}$  efflux, neurons were then reperfused in PSS to replenish  $\text{Ca}^{2+}$  stores and the sequence was repeated.  $\text{Ca}^{2+}$  efflux from the ER is indicated by a shift in the 340/380-nm ratio of Fura-2 fluorescence emittance. (F, G) *dt27* sensory neurons showed reduced ER calcium efflux relative to WT sensory neurons as indicated by a reduction in maximum peak amplitude following caffeine administration; Student's *t* test, \*\* $p < 0.01$ ,  $n = 61$ –63.  $\text{Ca}^{2+}$  reuptake was normal in both WT and *dt27* sensory neurons as indicated by the second peak response. The experimental paradigm in E was repeated in (H) WT and (I) *dt27* sensory neurons, with the exception that between the first and second sequences the neurons were perfused with thapsigargin (Thaps) for 180 s to block  $\text{Ca}^{2+}$  reuptake by SERCA pumps. Following SERCA inhibition, caffeine stimulation did not result in  $\text{Ca}^{2+}$  mobilization, confirming that caffeine was indeed stimulating efflux from ER stores alone and that all  $\text{Ca}^{2+}$  in the ER was effluxed following stimulation with caffeine.

## MATERIALS AND METHODS

### Reagents

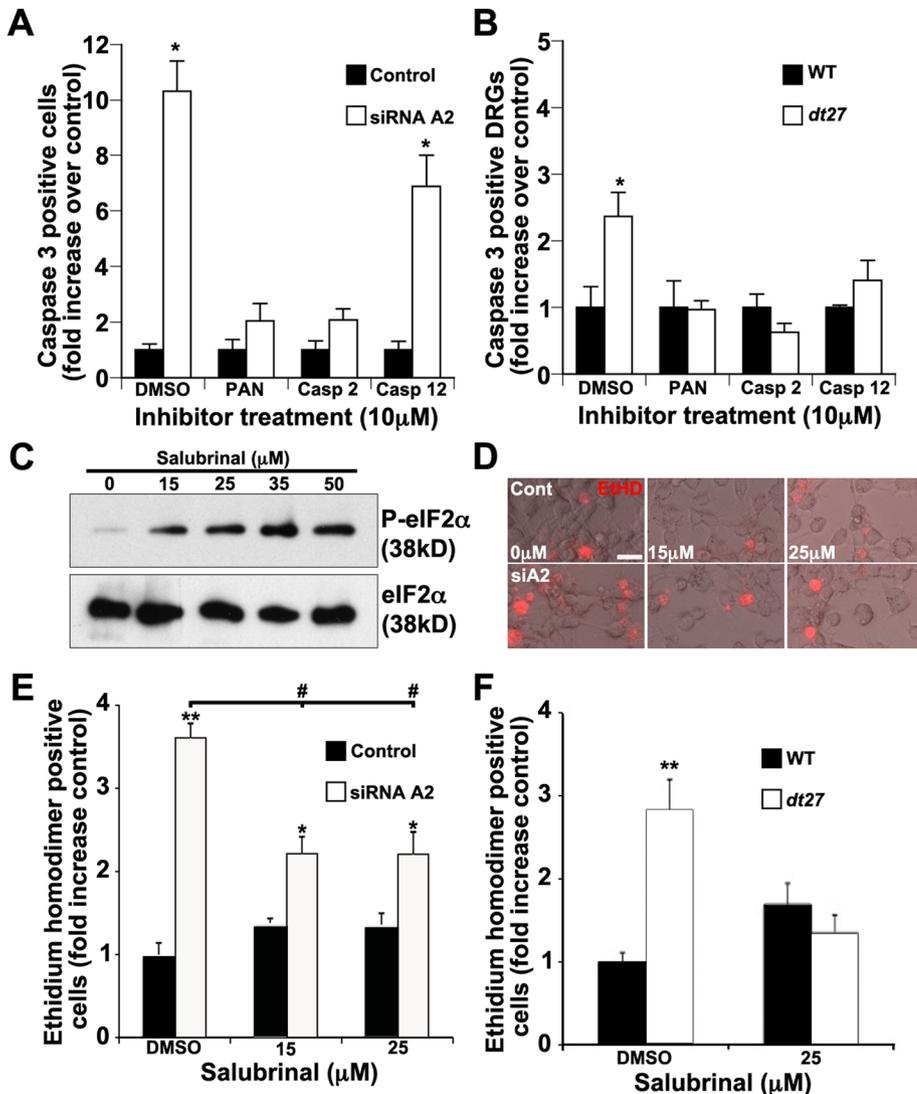
All chemicals were purchased through Sigma-Aldrich (St. Louis, MO), and all cell culture reagents were obtained from Invitrogen (Burlington, Canada), except where indicated.

### Animals and cell culture

The *dt27* and *dt<sup>Tg4</sup>* mutant mice and control littermates were used at prephenotype (P4 and P10) or phenotype stage (P15). The generation of these lines and characterization of the mutation were described previously (Kothary *et al.*, 1988; Brown *et al.*,

1995; Pool *et al.*, 2005). The onset of phenotype was generally assessed by the appearance of clasping of hind limbs after the mice were picked up by the tails. *dt27* and *dt<sup>Tg4</sup>* mice were genotyped by PCR amplification of genomic tail DNA. DRG tissue sections of the lumbar spinal cord region were prepared as previously described (Young and Kothary, 2008). All animal procedures were performed in accordance with institutional guidelines (Animal Care and Veterinary Services and Ethics, University of Ottawa).

Cos-1 cells (an African green monkey kidney fibroblast-like cell line obtained from American Type Culture Collection, Manassas,



**FIGURE 8:** Inhibition of ER stress and the associated caspase cascade rescues dystonin-a2-deficient neurons from death. (A) F11 neuronal cells were screened for caspase 3 activation following knockdown of dystonin-a2 in the presence of various caspase inhibitors using the FLICA assay. Pan-caspase inhibition or inhibition of caspase 2 but not caspase 12 prevented downstream caspase 3 activation in F11 neuronal cells; ANOVA, post hoc Dunnett's *t* test,  $*p < 0.05$ . (B) In P15 primary sensory neurons from *dt*<sup>27</sup> mice subjected to the same assay, pan-caspase inhibition in addition to inhibition of either caspase 2 or caspase 12 prevented downstream caspase 3 activation; ANOVA, post hoc Dunnett's *t* test,  $*p < 0.05$ ,  $n = 9$ . (C) Western analysis of F11 neuronal cells treated with various concentrations of salubrinal confirmed that salubrinal inhibits downstream UPR signaling by preventing dephosphorylation of eIF2 $\alpha$ . Fold change in ethidium homodimer incorporation showed that whereas dystonin-a2 depletion causes a significant increase in death (ANOVA, post hoc Tukey,  $**p < 0.01$ ,  $n = 9$ ), salubrinal treatment at 15 and 25  $\mu\text{M}$  significantly reduces death resulting from depletion of dystonin-A2 in F11 neuronal cells relative to vehicle (DMSO)-treated cells (D, E) (scale bars, 10  $\mu\text{m}$ ; ANOVA, post hoc Tukey,  $\#p < 0.05$ ). (F) Measurements of fold change in ethidium homodimer incorporation indicate that 25  $\mu\text{M}$  salubrinal treatment rescues *dt*<sup>27</sup> primary sensory neurons from death; Student's *t* test,  $**p < 0.01$ ,  $n = 18$ –20.

VA) and F11 cells (a fusion of embryonic rat DRG cells with a mouse neuroblastoma cell line, kindly supplied by Paul Albert, University of Ottawa) were maintained in DMEM + 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/antimycotic. Cells were passaged at ~70% confluency in 10-cm plastic Petri dishes and plated onto glass coverslips for use in immunofluorescence assays. Cell transfections were performed using Lipofectamine 2000 (Invitrogen), according to the manufacturer's directions.

dystonin-a1 and dystonin-a2, isoform-specific nucleotides between 2310 and 2380 (accession number AF396878) for dystonin-a1 and nucleotides between 1 and 661 (accession number: DQ02331) for dystonin-a2 were chosen for the development of siRNAs. The following groups of siRNA sequences were efficacious in knocking down dystonin-a1 and dystonin-a2 expression, respectively: group A (sense 5'-3') ACAUGUACGUGGAGGAGCAAtt; (antisense 5'-3') UGCUCC-UCCACGUACAUGUag, (sense 5'-3') CCGCCUACAUGUACGUGGAtt

### Primary culture of sensory neurons

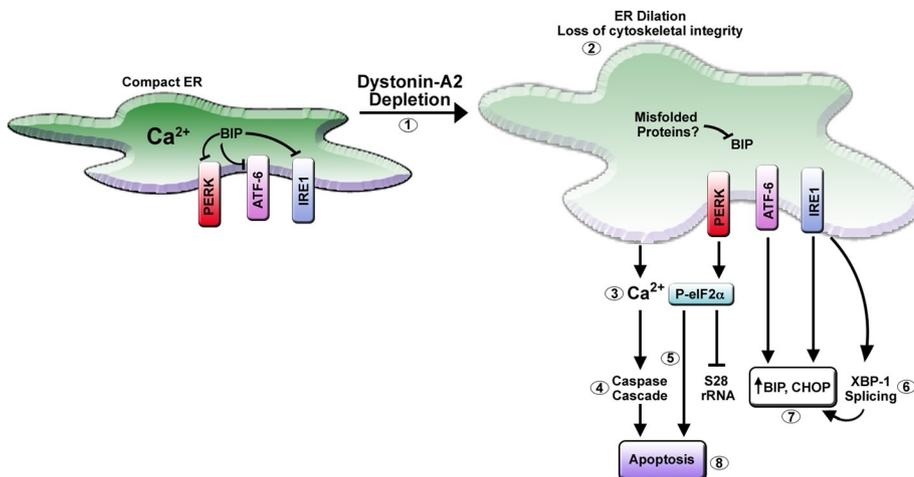
Spinal columns were removed from P4, P10, or P15 mice and transferred to a dissection microscope. Approximately 40 DRGs were isolated per mouse and subsequently digested for 15 min each with collagenase A (Roche, Laval, Canada) and papain (Worthington, Lakewood, NJ) solutions. DRG neurons were dissociated with flame-polished glass Pasteur pipettes and seeded onto 12-mm laminin-2 (Millipore, Billerica, MA)-coated coverslips at a density of 50,000 in DMEM with 10% FBS and 1% penicillin/streptomycin. Cells were placed in a 37°C tissue culture incubator under 8.5% CO<sub>2</sub>. The following day, the media was changed to neuronal maintenance media (DMEM base, 0.5% FBS, 2% B27, 1% GlutaMax, 16  $\mu\text{g/ml}$  putrescine, 400  $\mu\text{g/ml}$  thyroxine, 400  $\mu\text{g/ml}$  triiodothyronine, 6.2 ng/ml progesterone, 5 ng/ml sodium selenite, 100  $\mu\text{g/ml}$  bovine albumin serum, 5  $\mu\text{g/ml}$  bovine insulin, 50  $\mu\text{g/ml}$  holotransferrin) supplemented with 200 ng/ml nerve growth factor and 1  $\mu\text{M}$  5-fluoro-2'-deoxyuridine. A three-fourths media change was carried out every other day, up until day 6 of culture, when calcium-imaging experiments were performed.

### Sample preparation for electron microscopy

Samples for electron microscopy were prepared as previously described (De Repentigny *et al.*, 2011). Briefly, *dt*<sup>27J</sup> ( $n = 4$ ) and WT ( $n = 4$ ) mice were anesthetized at P5, P10, or P15 via intraperitoneal injection of tribromoethanol (Avertin). Mice were perfused transcardially with 3 ml of phosphate-buffered saline (PBS), followed by 10 ml of Karnovsky's fixative (4% paraformaldehyde [PFA], 2% glutaraldehyde, and 0.1 M cacodylate buffer in PBS, pH 7.4). The lumbar region of the spinal cord was dissected and DRGs collected under a stereomicroscope. Ultrathin sections (70 nm) were placed on a 300-mesh copper specimen grid, counterstained in 5% uranyl acetate and Reynold's lead citrate, and then observed by transmission electron microscopy.

### siRNA and RT-PCR

Custom siRNAs were developed to target specific dystonin isoforms. To knock down



**FIGURE 9:** Proposed model of apoptotic signaling initiated by depletion of dystonin-a2 in sensory neurons. 1) Schematic representation of the events that occur following depletion of dystonin-a2 in sensory neurons. 2) Depletion of dystonin-a2 results in a loss of cytoskeletal integrity that culminates in dilation of the ER. 3) Perturbed ER homeostasis leads to a decrease in the steady-state levels of  $\text{Ca}^{2+}$  in the ER. 4) The associated rise in free intracellular  $\text{Ca}^{2+}$  likely results in activation of an ER-dependent caspase cascade, inhibition of which can maintain cell viability. 5) Blockade of downstream eIF2 $\alpha$  signaling with salubrinal partially rescues neurodegeneration following dystonin-a2 depletion, implicating PERK-mediated eIF2 $\alpha$  signal transduction in the apoptotic cascade. 6) Enhanced XBP1 splicing likely coupled with activation of ATF-6 and IRE1 promotes expression of protein chaperones (7) BiP and CHOP in an attempt to maintain ER function. 8) The inability to rescue ER structural integrity culminates in DNA cleavage and programmed cell death.

(antisense 5'–3') UCCACGUACAUGUAGGCGGcg; group B (sense 5'–3') CAAGCAUGAGAGAUCCAAAt; (antisense 5'–3') UUUGGAUCUCUCAUGCUUGgg; (sense 5'–3') CUUCCUCUUGUUGCUCCUGtt; (antisense 5'–3') CAGGAGCAACAAGAGGAAGag. The siRNA target sequences were conserved between mouse and rat. All siRNAs (Silencer Select Custom Designed siRNA) were obtained from Applied Biosystems/Ambion (Austin, TX) and compared with the effects of scrambled controls matched for GC content. F11 and Cos-1 cells were transfected with siRNA using Lipofectamine 2000. Knockdown was assessed at the transcript and protein level. Total RNA was collected from F11 cells using RNeasy Mini Kit (Qiagen, Toronto, Canada) according to manufacturer's protocol. For RT-PCR, QuantiTect Reverse Transcription Kit (Qiagen) was used, following manufacturer's protocol. cDNAs encoding dystonin-a1, dystonin-a2, and actin (control) derived from mouse were PCR amplified as previously described (Pool *et al.*, 2005). For assessment of protein knockdown, Cos-1 cells were cotransfected with siRNA and recombinant dystonin proteins (dystonin-a2-YFP and Nterm-dystonin-a1-GFP) using Lipofectamine 2000. Fluorescence intensity of recombinant dystonin proteins was used to assess siRNA knockdown efficiency.

### Recombinant proteins

The dystonin-a1-GFP construct (GFP-Nterm1) was previously described (Young *et al.*, 2003). The dystonin-a2-YFP construct was designed and cloned by Kevin Young while a member of the Kothary lab. The 5' N-terminal region of the dystonin-a2 isoform (a2Nterm-myc/his; Young and Kothary, 2008) and the 3' end of an expressed sequence tag clone (GenBank accession A1526522; Young *et al.*, 2006) were ligated in frame into a pEYFP-N1 vector. The reporter plasmid pHA-XBP1u-GFP was a gift from John Ngsee of the Ottawa Hospital Research Institute and was previously described (Yu *et al.*, 2006). Calreticulin-YFP and Golgi-YFP were gifts from Xiaohui Zha of the Ottawa Hospital Research Institute.

### Cell viability

Dead cells were identified by uptake of ethidium homodimer (2  $\mu\text{M}$ ) as a result of loss of membrane integrity. Cells were then visualized with a Zeiss Axiovert 200m epifluorescence microscope under 10 $\times$  objective (Achromplan 0.25) equipped with an AxioCam HRM digital camera and AxioVision 4.6 software (Zeiss, Toronto, Canada).

### Fluoro-Jade B staining

Fluoro-Jade B (Millipore) staining was performed according to the method of Schmued *et al.* (1997). Briefly, 12- $\mu\text{m}$  DRG sections were fixed with 4% PFA dried on a slide warmer at 45°C. The tissue was then subjected to a series of washes (100% ethanol, 3 min; 70% ethanol, 1 min; ddH<sub>2</sub>O, 1 min; 0.06% potassium permanganate, 15 min; double-distilled water, 1 min; 0.001% Fluoro-Jade in 0.1% acetic acid, 20 min; 3 $\times$  double-distilled water, 1 min; and xylene, 1 min; and then coverslipped with Permount [Fisher Scientific, Ottawa, Canada]). Sections were analyzed with a Zeiss Axiovert 200m epifluorescence microscope under 10 $\times$  objective (Achromplan 0.25) equipped with an AxioCam HRM digital camera and AxioVision 4.6 software.

### TUNEL

Cells/tissue was washed in PBS and permeabilized in ice-cold 0.1% sodium citrate/0.1% Triton X-100 for 5 min, followed by 2 min in 2:1 ethanol:acetic acid on ice. Samples were rinsed for 2 min in PBS and incubated for 1 h at 37°C with FITC-labeled dUTP in terminal deoxynucleotidyl transferase (TdT) buffer (30 mM Tris-HCl, pH 7.2, 140 mM sodium cacodylate, and 1 mM cobalt chloride) and TdT according to the protocol provided by the manufacturer (Roche). Negative controls included sections incubated with fluorescein isothiocyanate-labeled dUTP in the absence of TdT. Cells were washed in PBS, mounted in fluorescence mounting media (Dako, Burlington, ON), and analyzed with a Zeiss Axiovert 200m epifluorescence microscope under 10 $\times$  objective (Achromplan 0.25) equipped with an AxioCam HRM digital camera and AxioVision 4.6 software.

### Caspase activity assay

Caspase activity in live cells was measured using fluorescein (caspase 12, fluorescein isothiocyanate-ATAD-fluoromethyl ketone [FMK]; Abcam, Cambridge MA) and fluorescence-labeled inhibitor of caspase activity (FLICA; caspase 8, carboxyfluorescein [FAM]-LETD-FMK; caspase 2, FAM-VDVAD-FMK; and caspase 3/7, FAM-DEVD-FMK; ImmunoChemistry Technologies, Bloomington, MN) assays. Fluorescein/FLICA assays were performed using a modified protocol from the manufacturers' instructions. Briefly, 1.67  $\mu\text{l}$  of 30 $\times$  fluorescein/FLICA reagent (diluted in PBS) was added to each well of a 96-well plate. Cells were incubated for 1 h at 37°C and 5% CO<sub>2</sub>. Cells were washed twice in PBS and analyzed with a Zeiss Axiovert 200m epifluorescence microscope under 10 $\times$  objective (Achromplan 0.25) equipped with an AxioCam HRM digital camera and AxioVision 4.6 software. Phase contrast and fluorescence photos were taken of six random fields per well for three of four replicate wells in duplicate or triplicate experiments. The mean number of cells with active caspases at each time point was determined, and data were then

standardized against scrambled or WT controls where appropriate to determine fold change in caspase activity attributable to loss of dystonin. Functional dependence of downstream caspase activation on active caspases 2 and 12 or other caspase activation (PAN) was assessed. F11 or primary sensory neurons were treated with either the caspase 2 inhibitor Z-VAD-FMK (50  $\mu$ M), the caspase 12 inhibitor Z-ATAD-FMK (50  $\mu$ M), or the PAN caspase inhibitor Z-VAD-FMK. Caspase 3 activation was then assessed as described.

### Immunocytochemistry

Cultures were fixed in 4% PFA prior to antigenic labeling. Primary antibodies used were rabbit polyclonal anti-cleaved caspase 3 (1:1000; Cell Signaling Technology, Beverly, MA), mouse monoclonal anti-caspase 2 (1:500; EMD Biosciences, San Diego, CA), and mouse monoclonal anti-CHOP (1:1000; Cell Signaling Technologies). Secondary antibodies used were anti-mouse Alexa 488 (1:2000; Molecular Probes, Invitrogen), anti-mouse Alexa 555 (1:2000; Molecular Probes), and anti-rabbit Alexa 488 (1:2000; Molecular Probes). Antibodies were diluted in antibody buffer (PBS, 0.3% Triton X-100, 3% BSA). Where 4',6-diamidino-2-phenylindole (DAPI) staining is indicated, samples were incubated in DAPI stain (0.2  $\mu$ g/ml in PBS) for 10 min and washed three times in PBS for 5 min. Samples were mounted in fluorescence mounting media (Dako) and analyzed a Zeiss Axiovert 200m epifluorescence microscope under either a 10 $\times$  objective (Achromplan 0.25) or a 40 $\times$  objective (Achromplan 1.3 Oil) equipped with an AxioCam HRM digital camera and AxioVision 4.6 software. Where colocalization studies were performed, F11 neuronal cells treated with either scrambled siRNA or siRNA targeted against dystonin-a2 were analyzed with an LSM 510 meta confocal microscope equipped with an EC Plan-Neofluar 40 $\times$ /1.30 Oil DIC M27 objective using Zen 8.0 software (Zeiss). Using the colocalization module of Zen 8.0, we compared the total area of caspase 2 staining per the cell with the area of caspase 2 staining that colocalized with either calreticulin-YFP or Golgi-YFP within that same cell. The area of colocalization was then calculated as the percentage colocalized area over the total area of caspase 2. Twelve cells from three different experiments were analyzed per condition.

### Western analysis

Proteins were isolated in RIPA buffer (10 mM PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 30  $\mu$ l/ml aprotinin, 10 mM Na orthovanadate, 100  $\mu$ l/ml phenylmethylsulfonyl fluoride). Protein samples (30  $\mu$ g) were separated by SDS-PAGE under reducing conditions. Western analyses were performed using primary mouse monoclonal anti-caspase 2 (1:500; BD Transduction Laboratories, Lexington, KY), mouse monoclonal anti-CHOP (1:1000, Cell Signaling Technology), polyclonal anti-binding protein (BiP, 1:1000; Cell Signaling Technology), rabbit polyclonal anti-phospho-eIF2 $\alpha$  (1:1000; Cell Signaling Technology), rabbit polyclonal anti-eIF2 $\alpha$  (1:1000; Cell Signaling Technology), rabbit polyclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Abcam), and mouse monoclonal anti-BIII-tubulin (1:2000, E7 clone; Hybridoma Bank, Iowa City, IA). Owing to low protein yields from DRGs, analysis of ER stress (Figure 6 and Supplemental Figure S2) was performed using pooled DRGs from three WT and three *dt<sup>27</sup>* mice per sample. A 40- $\mu$ g protein sample was loaded thereafter. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated anti-mouse immunoglobulin G (IgG, 1:2000; Bio-Rad, Hercules, CA) and HRP-conjugated anti-rabbit IgG (1:2000; Bio-Rad). Immunoreactive bands were visualized using SuperSignal West Pico (MJS BioLynx, Brockville, Canada).

Quantification of protein expression levels was performed by densitometric analysis of individual bands using ImageJ analysis software (National Institutes of Health, Bethesda, MD). At least three Western blots from different experiments were evaluated by densitometry and normalized to a protein standard (actin, tubulin, or GAPDH) for all Western blots depicted.

### Ratiometric measurement of intracellular Ca<sup>2+</sup> using Fura-2AM in primary sensory neurons

The measurement of intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) was performed using the ratiometric dye Fura-2AM based on previously published protocol (Jiang *et al.*, 2005). Briefly, P15 sensory neuron cultures or F11 neuronal cells were grown on 2-mm laminin-2 (Millipore)-coated coverslips (as described) and loaded with 5  $\mu$ M Fura-2AM (Molecular Probes) and 0.02% pluronic acid in neuronal maintenance media and placed in a 37°C tissue culture incubator under 8.5% CO<sub>2</sub> for 30 min. Sensory neuron cultures were then rinsed with PSS Mg<sup>2+</sup> buffer (2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.2, 140 mM NaCl, 5 mM KCl, 2.3 mM CaCl<sub>2</sub>, and 10 mM glucose) and stabilized in the same buffer for 5 min and then placed in a microperfusion chamber, where Fura-2AM intensities were measured by the Northern Eclipse Digital Ratio Image System (EMPIX, Mississauga, Canada) with an Axiovert 200 camera and light source (Zeiss). After a region was chosen, Fura-2 fluorescence was measured at 510-nm emission with 340/380-nm dual excitation selected by a DG-5 system (Sutter Instrument Company, Novato, CA). [Ca<sup>2+</sup>]<sub>i</sub> concentration was determined via the ratio of fluorescence intensity between the two excitation wavelengths of 340/380 nm of Fura-2. The basal level of [Ca<sup>2+</sup>]<sub>i</sub> was recorded for 100 s in PSS buffer, followed by a 30-s perfusion of calcium-free Tyrode solution (9 mM NaCl, 12.5 mM KCl, 3 mM MgCl<sub>2</sub>, 30 mM glucose, 25 mM HEPES, 1 mM ethylene glycol tetraacetic acid, pH 7.4), followed by a 30-s perfusion of Tyrode solution plus caffeine (20 mM). These perfusions were repeated a second time or following 180 s of perfusion with thapsigargin (20 nM) in PSS.

### Statistical analysis

Data were analyzed using Student's *t* test or factorial analysis of variance (ANOVA) as applicable using InStat, version 3.0 (GraphPad Software, La Jolla, CA). Following detection of a statistically significant difference in a given series of treatments by ANOVA, post hoc Dunnett's *t* tests or Tukey tests were performed where appropriate. *p* < 0.05 was considered statistically significant (shown as either \* or #); *p* < 0.01 was considered highly statistically significant (shown as \*\*).

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