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# The Vacuolar H+-ATPase Mediates Intracellular Acidification Required for Neurodegeneration in *C. elegans*

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## Summary

**Numerous studies implicate necrotic cell death in devastating human pathologies such as stroke and neurodegenerative diseases [1, 2]. Investigations in both nematodes and mammals converge to implicate specific calpain and aspartyl proteases in the execution of necrotic cell death [2, 3]. It is believed that these proteases become activated under conditions that inflict necrotic cell death. However, the factors that modulate necrosis and govern the erroneous activation of these otherwise benign enzymes are largely unknown. Here we show that the function of the vacuolar H+-ATPase, a pump that acidifies lysosomes and other intracellular organelles, is essential for necrotic cell death in *C. elegans*. Cytoplasmic pH drops in dying cells. Intracellular acidification requires the vacuolar H+-ATPase, whereas alkalization of endosomal and lysosomal compartments by weak bases protects against necrosis. In addition, we show that vacuolar H+-ATPase activity is required downstream of cytoplasmic calcium overload during necrosis. Thus, intracellular pH is an important modulator of necrosis in *C. elegans*. We propose that vacuolar H+-ATPase activity is required to establish necrosis-promoting, acidic intracellular conditions that augment the function of executioner aspartyl proteases in dying cells. Similar mechanisms may contribute to necrotic cell death that follows extreme acidosis—for example, during stroke—in humans.**

## Results and Discussion

### The Vacuolar H+-ATPase Is Required for Necrotic Cell Death

The vacuolar H+-ATPase (V-ATPase) is a universal, multi-subunit proton pump that acidifies intracellular organelles and regulates cellular pH at the expense of ATP [4]. A series of 17 *vha* genes (vacuolar H+-ATPase), *spe-5*, and *unc-32* encode subunits of the V-ATPase in *C. elegans* (R. Weimer and E. Jorgensen, University of Utah, personal communication; P.D. Hartley, G.-d. Zhu, and S.W. L' Hernault, Emory University, personal communication; WormBase WS123, http://www.wormbase.org; see Table S1 for a list of *C. elegans* genes encoding V-ATPase subunits) [5–8]. We examined the involvement of V-ATPase in necrosis triggered by hyperactive MEC-4, an ion-channel subunit normally required for mechanotransduction in the six touch-receptor neurons of *C. elegans* [9, 10]. Mutations in *spe-5*, *unc-32*, and *vha-12* suppressed neurodegeneration in animals expressing a neurotoxic, gain-of-function *mec-4* allele (*u231* or *d*; dominant; see Figure 1A). We obtained similar cell-death suppression by RNAi-mediated knockdown of *vha-2*, *vha-10*, and *vha-12* (Figure 1A). In addition, we treated *mec-4(d)* animals with bafilomycin A1 and filomycin, two antibiotics that specifically inhibit the V-ATPase [11, 12]. Treatment ameliorated necrosis of the six touch-receptor neurons (Figure 1A). Survival of touch-receptor neurons was confirmed by the presence of fluorescent, $p_{mec\text{-}4}$GFP-expressing cells in adult animals (data not shown).

Suppression of *mec-4(d)*-induced cell death in the genetic backgrounds and under the conditions examined is not a consequence of a mere reduction in the toxic MEC-4(d) protein levels. We assayed the relative expression and stability of MEC-4 by utilizing reporter genes with both *Lac*Z and GFP fused at the carboxyl terminus of the full-length MEC-4 protein. Fusion-protein levels were not affected by manipulations that reduce V-ATPase activity [Figure 1B; the case with *unc-32(e189)* is shown as an example].

Next, we asked whether V-ATPase deficiency is generally protective against necrotic cell death. Gain-of-function (*d*) mutations in two otherwise unrelated genes, *deg-3*, which encodes the α-7 nicotinic acetylcholine-receptor $Ca^{2+}$-channel subunit, and *gsa-1*, which encodes the $G\alpha_s$ subunit, trigger necrotic degeneration of specific sets of neurons expressing the toxic variants [13–15]. Prolonged hypoxia, which is a condition of low oxygen availability and transpires in ischemic episodes and stroke, also induces necrotic cell death in the nematode [16]. Cell death inflicted by the toxic *deg-3(d)* allele and by overexpression of the hyperactivated $G\alpha_s$(Q227L) variant ($\alpha_s$*(gf)*) was suppressed in V-ATPase-deficient, *unc-32*, and *vha-12* mutant genetic backgrounds (Figure 1C). Furthermore, *unc-32* and *vha-12* mutations increased the survival of animals under hypoxic conditions (Figure 1D). Similarly, bafilomycin A1, which blocks the V-ATPase pump, ameliorated neurodegeneration and protected cells from hypoxia-induced death (Figures 1C and 1D). Expression of the *deg-3* and $\alpha_s$*(gf)* genes was not detectably reduced in these experiments. We conclude that V-ATPase activity is broadly required for neurodegeneration caused by deleterious mutations in a diverse set of genes, as well as under hypoxic conditions in *C. elegans*.

The single and double mutants we examined for neurodegeneration do not show any discernible growth defects. Furthermore, neurodegeneration in *C. elegans* is generally not affected by growth defects (P.S. and N.T., unpublished observations). Necrotic cell death triggered by *mec-4(d)* peaks during the L1 larval stage of *C. elegans*, with fewer vacuolated cells being observed thereafter [17]. We examined whether the apparent reduction in necrotic neuron number at the L1 stage of animals carrying mutations in V-ATPase subunits is the result of a delay in rather than suppression of cell death. We carried out a time-course analysis of necro-

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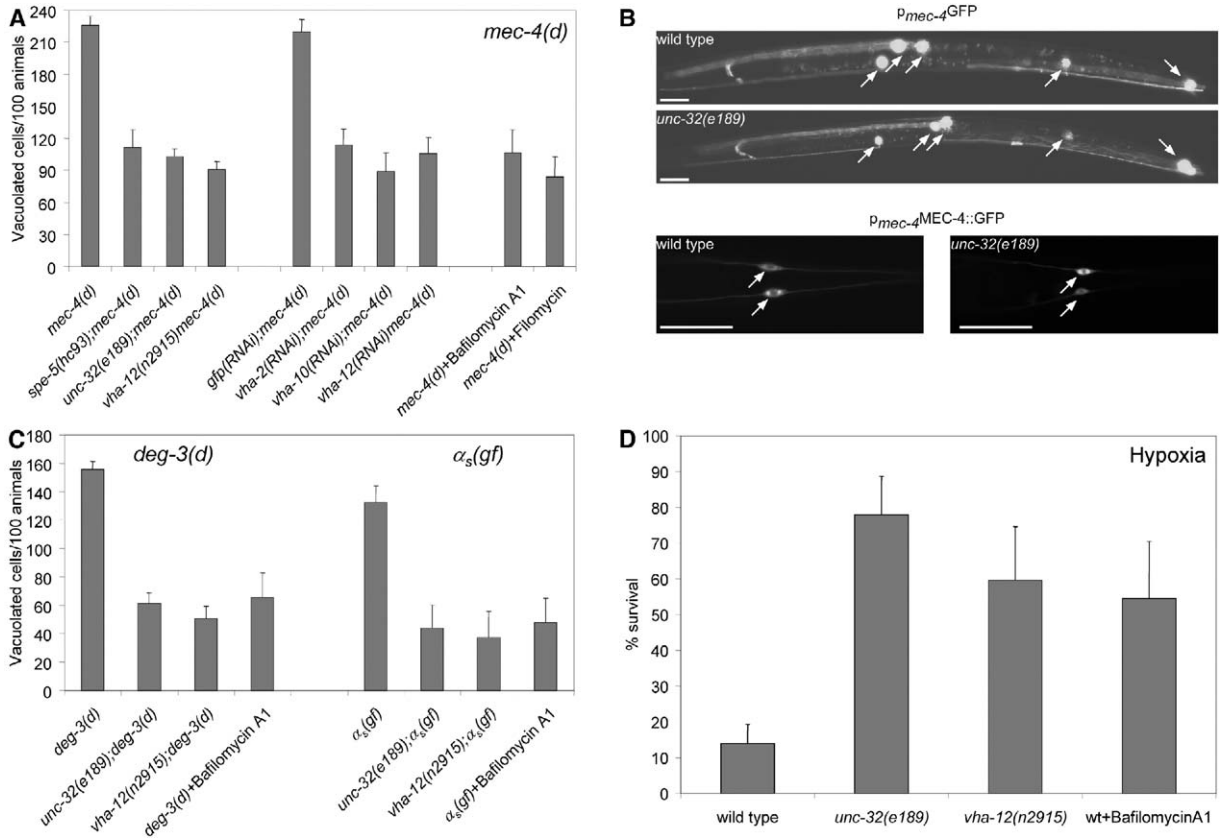


Figure 1. The Vacuolar H+-ATPase Is Generally Required for Neurodegeneration in *C. elegans*

(A) Number of vacuolated touch receptors, at the L1 stage, per 100 animals carrying the *mec-4(d)* allele in genetic backgrounds with reduced V-ATPase activity under RNAi with genes encoding V-ATPase subunits and after treatment with bafilomycin A1 and filomycin (n = 350, $p < 0.001$, unpaired t test). *mec-4(d)* animals show, on average, about 2.2 degenerating cells out of the six touch-receptor neurons expressing the *mec-4(d)* allele (see Supplemental Data). Wild-type nematodes show zero neurodegeneration. Efficacy of RNAi was assessed as described in the Supplemental Data section. Error bars represent standard deviations.

(B) Expression of GFP in touch-receptor neurons, either driven solely by the *mec-4* promoter (top two panels; all six cells are visible) or fused at the end of the full-length MEC-4 protein (bottom two panels; only the two tail neurons are shown). The V-ATPase deficiency in *unc-32(e189)* mutant animals does not affect *mec-4* expression or stability. White bars denote approximately 50 μm. Error bars represent standard deviations.

(C) For *deg-3(d)*- and $\alpha_s$*(gf)*-induced necrosis, bars denote vacuolated PVC interneurons per 100 L1 larvae (n = 250, $p < 0.001$, unpaired t test). White arrows point to touch-receptor neurons. Error bars represent standard deviations.

(D) Hypoxic death is suppressed by reduced V-ATPase activity. The percentage of animals that survive near-lethal treatment with sodium azide ($NaN_3$; n = 200, $p < 0.001$, unpaired t test). Sodium azide inhibits the activity of respiratory chain electron transport complex IV (cytochrome c oxidase) and simulates hypoxia.

sis during all four *C. elegans* larval stages (L1–L4) and have not observed any shift in the cell-death peak to later developmental stages; such a shift which would indicate a delay in necrosis (Figure 2).

## Cytoplasmic pH Drops in Cells Undergoing Necrosis

We examined whether disruption of the lysosomal system during neurodegeneration affects pH homeostasis in the cytoplasm. We monitored cytoplasmic pH changes during necrotic cell death by utilizing two pH-sensitive GFP variants (superecliptic and ratiometric pHluorins). Under excitation by incident photons with a wavelength of 476 nm, superecliptic pHluorin (SepHluorin) emission at 515 nm is abolished after a shift to low pH. In contrast, ratiometric pHluorin (RmpHluorin) emission increases under acidic conditions [18, 19]. We quantified fluorescence of SepHluorin and RmpHluorin expressed specifically in the six touch-receptor neurons of wild-type and *mec-4(d)* mutant animals. SepHluorin emission was quenched, whereas RmpHluorin emission intensity was increased in dying neurons of *mec-4(d)* mutants, compared to wild-type (Figure 3). These observations indicate that cytoplasmic acidification accompanies neurodegeneration. Is the V-ATPase required for cytoplasmic acidification during necrosis? We examined pH changes in touch-receptor neurons of *vha-12mec-4(d)* double mutants, where V-ATPase activity is compromised. The development of acidic intracellular conditions was alleviated in these animals (Figure 3). We conclude that cytoplasmic pH drops in cells undergoing necrosis and that necrosis-associated acid-

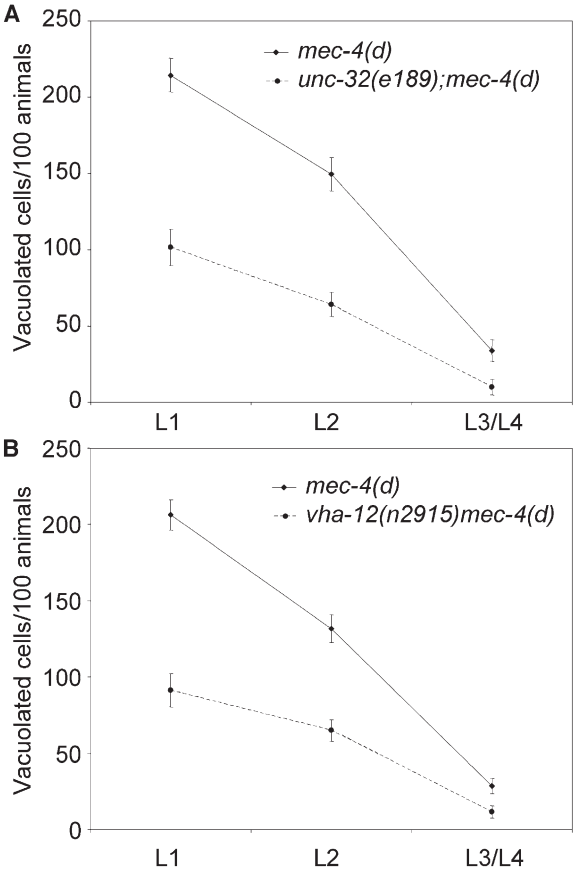


Figure 2. Time-Course Analysis of Necrotic Cell Death, Inflicted by *mec-4(d)*, in Mutant V-ATPase Genetic Backgrounds

(A) In *unc-32(e189)* mutant animals. The number of vacuolated touch receptors, at the larva stages indicated, per 100 animals is graphed (n = 200, $p < 0.001$, unpaired t test).

(B) In *vha-12(n2915)* mutant animals. Measurements with single *mec-4(d)* mutants are included for comparison.

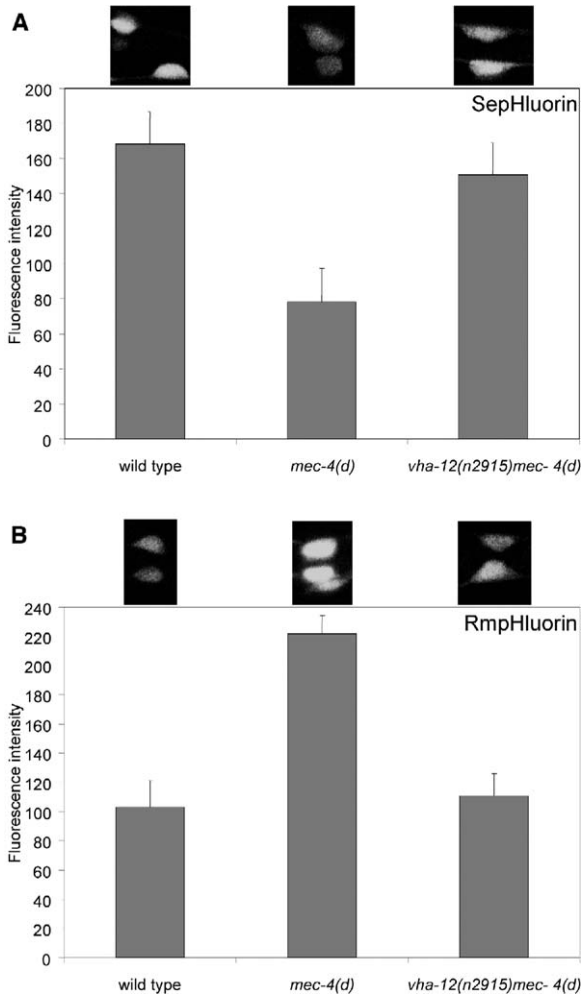


Figure 3. The Vacuolar $H^+$-ATPase Is Required for Cytoplasmic Acidification in Cells Undergoing Necrosis

(A) SepHluorin emission intensity at 515 nm is decreased in degenerating touch-receptor neurons of *mec-4(d)* animals compared to the wild-type. SepHluorin fluorescence is restored in neurons of *vha-12(n2915)mec-4(d)* double mutants, indicating that cytoplasmic acidification is alleviated.

(B) RmpHluorin exhibits the opposite behavior under identical conditions: Emission at 515 nm is increased in dying neurons of *mec-4(d)* animals, whereas no fluorescence enhancement is observed in neurons of *vha-12(n2915)mec-4(d)* double mutants. Scale bars represent average fluorescence intensity from three independent trials (8-bit scale; at least 150 touch-receptor-neuron images processed; $p < 0.001$, unpaired t test).

Representative images of PLM neurons expressing either the superecliptic or the ratiometric pHluorin are included above the graphs. Detailed information on fluorescence data collection and processing are provided in the Supplemental Data section. Error bars represent standard deviations.

ification requires the V-ATPase. Conditions that hinder intracellular acidification diminish cell death, suggesting that low cytoplasmic pH is a causative factor in necrosis rather than a mere consequence of cellular destruction. Ensuing cytoplasmic acidification may promote cell death by enhancing the activity of destructive, low-pH-dependent proteases that dismantle the cell.

### The Vacuolar $H^+$-ATPase Is Required Downstream of Cytoplasmic Calcium Elevation to Mediate Neurodegeneration

Recent studies suggest that release of $Ca^{2+}$ stores from the endoplasmic reticulum (ER) to the cytoplasm contributes to neurodegeneration initiated by hyperactive MEC-4 or $G\alpha_s$ [20]. Forced release of ER $Ca^{2+}$ by treatment with thapsigargin, a compound that also inhibits SERCA (an ER $Ca^{2+}$-reuptake pump) and leads to a net cytoplasmic calcium ($[Ca^{2+}]_i$) increase, induces necrotic cell death in *C. elegans* [20, 21]. We examined whether V-ATPase activity is required for thapsigargin-induced cell death. Mutations in *unc-32* and *vha-12* suppressed necrotic cell death in animals treated with thapsigargin. We obtained similar cell-death suppression by RNAi-mediated knockdown of *vha-10* (Figure 4A).

Perturbation of ER calcium homeostasis is known to interfere with necrotic cell death in *C. elegans* [20, 21]. Loss-of-function mutations in *crt-1*, a gene encoding calreticulin, a calcium binding ER chaperone, suppress

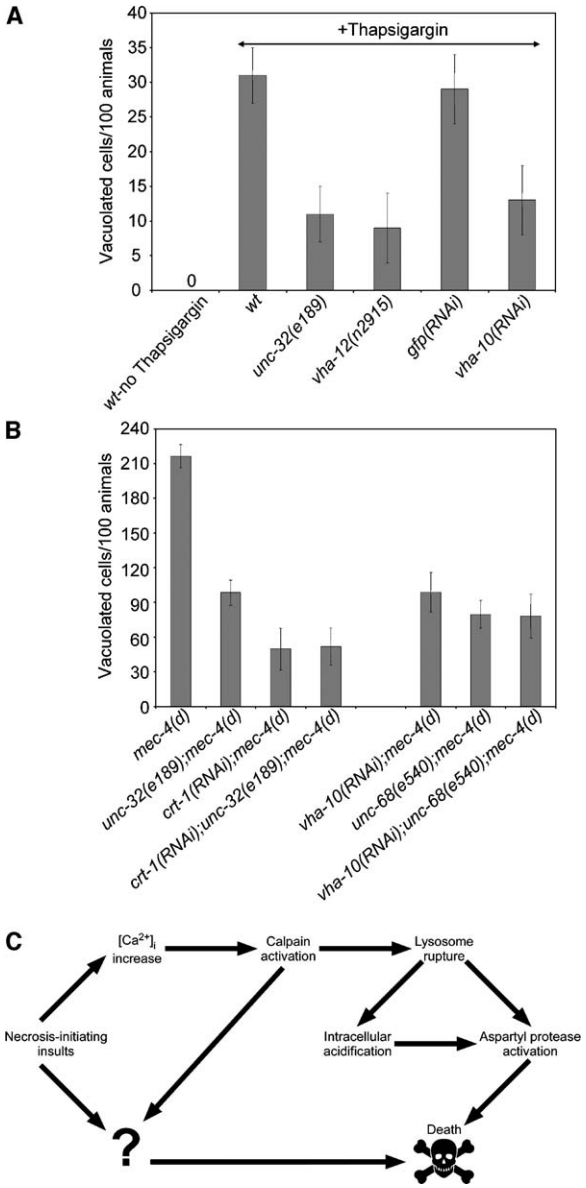


Figure 4. The Vacuolar H$^+$-ATPase Is Required Downstream of ER Calcium Release to Facilitate Necrotic Cell Death

(A) Thaspigargin-induced cell death is suppressed by conditions that diminish V-ATPase activity. Vacuolated cells per 100 L1 progeny of animals treated with thaspigargin (n = 120, $p < 0.001$, unpaired t test). Error bars represent standard deviations.

(B) Lack of synergism between conditions that affect V-ATPase function and ER calcium release during neurodegeneration. Number of vacuolated touch receptors, at the L1 stage, per 100 animals carrying the *mec-4(d)* allele in the genetic backgrounds indicated (n = 250, $p < 0.001$, unpaired t test). The efficacy of RNAi was assessed as described in the Supplemental Data section. Error bars represent standard deviations.

(C) Working model for a necrotic-cell-death pathway in *C. elegans*. Death signals modulate $[Ca^{2+}]_i$ levels, which are sensed by calcium-activated calpain proteases. In turn, calpains provoke lysosomal rupture. The consequent intracellular acidification, which requires the V-ATPase, potentiates a specific set of executioner aspartyl proteases that dismantle the cell. We note that residual neurodegeneration in genetic backgrounds with protease and V-ATPase deficiency indicates that additional mechanisms contribute to cell death (denoted by the question mark). Triggering input to these pathways could be provided by intracellular calcium overflow or directly by cell-death-initiating insults.

necrosis induced by *mec-4(d)* [20]. In addition, mutations in *unc-68*, encoding a ryanodine-receptor ER calcium-release channel, ameliorate *mec-4(d)*-induced necrosis by diminishing release of ER calcium stores [20]. We examined the requirement for V-ATPase activity in neurodegeneration under conditions of compromised calcium release from the ER. Mutations in *unc-32* and RNAi-mediated knockdown of *vha-10* do not further enhance necrosis suppression by calreticulin or ryanodine-receptor mutants (Figure 4B). The totality of our observations suggests that, although mobilization of ER calcium stores is required for the buildup of noxious $[Ca^{2+}]_i$ levels in the cytoplasm, the V-ATPase contributes downstream of $[Ca^{2+}]_i$ signaling to facilitate cell death. This conclusion is further corroborated by the requirement for V-ATPase activity in neurodegeneration induced by hyperactive alleles of *deg-3*, a gene encoding an acetylcholine-receptor $Ca^{2+}$ channel (Figure 1C).

## Conclusions

We document a general requirement for vacuolar H$^+$-ATPase, a pump that acidifies lysosomes and other intracellular organelles, in necrotic cell death in the nematode. Furthermore, we demonstrate that reduced vacuolar H$^+$-ATPase activity or alkalization of acidic endosomal/lysosomal compartments by weak bases has a neuroprotective effect against necrosis initiated by a diverse set of genetic, chemical, and environmental insults. Our analysis shows that an acute drop of cytoplasmic pH accompanies necrosis in *C. elegans*. Acidification requires the function of the vacuolar H$^+$-ATPase.

What is the origin of cytoplasmic acidification that accompanies necrosis? We hypothesize that strong ionic imbalance, which triggers necrotic cell death, may overwhelm the pH-buffering capacity of the cell. Recent investigations in *C. elegans* and primates demonstrated that specific calpain proteases are required for necrotic cell death [2, 22]. Calpains are a diverse class of intracellular papain-like cysteine proteases that require calcium for activation [23, 24]. These enzymes become activated by the abrupt increase of intracellular calcium ($[Ca^{2+}]_i$) concentration, which signals the initiation of necrosis (reviewed in [21]). In primate hippocampal neurons, degeneration after acute ischemia is accompanied by $[Ca^{2+}]_i$ elevation and concomitant calpain activation. It is hypothesized that, in addition to their other functions, calpains compromise the integrity of lysosomal membranes and thereby cause leakage of their acidic contents into the cytoplasm. In agreement with this hypothesis, activated calpain proteases localize to disrupted lysosomal membranes [22, 25].

Our data, together with previous findings, suggest that diverse death-triggering stimuli converge to initially elicit cytoplasmic calcium elevation and concomitant calpain activation. In addition to neutral, calcium-regulated calpains, specific acidic pH-activated aspartyl proteases are required for neurodegeneration. We propose that the V-ATPase pump underlies the develop-

ment of acidic intracellular conditions during necrosis. Diverse death-triggering stimuli converge to, in part, elicit calpain-mediated rupture of lysosomes and release of their acidic contents and catabolic enzymes into the cytoplasm. Concomitant cytoplasmic acidification activates or further boosts the activity of low pH-dependent aspartyl proteases and other hydrolases and thus contributes to cell destruction (Figure 4C). The collapse of intracellular pH homeostasis is also likely to interfere with critical cellular processes, adding insult to injury and rendering the cell incapable of sustaining life. In line with this model, we find that V-ATPase activity is important for cell death triggered by cytoplasmic calcium overload caused by ER calcium release. These observations indicate that acidification is a factor required downstream of cytoplasmic calcium elevation and calpain activation in the necrotic pathway. We did not achieve complete or near-complete blockage of neurodegeneration by manipulations that reduce V-ATPase activity. Our results are consistent with previous observations showing that calpain or cathepsin protease deficiency does not completely block necrotic cell death [2]. Therefore, it is likely that additional mechanisms contribute to cellular destruction during necrosis. Genetic suppressor screenings should in principle reveal more players in the process.

Elucidation of the events that precipitate collapse of cellular pH homeostasis during neurodegeneration in *C. elegans* may provide insight into similar pathologies in humans. Interestingly, acidosis accompanies necrotic cell death after stroke in mammals [26, 27], and the lysosomal-degradation system was found to be upregulated in neurons of patients with Alzheimer's disease [28]. Recent studies of postmortem human brains associate neuronal pH alterations with several pathological states [29]. In addition, extracellular acidosis, which develops during stroke in mammals, contributes to neurodegeneration by activating acid-sensing, calcium-permeable ion channels (acidotoxicity) [30, 31]. Our findings suggest that the molecular mechanisms underlying necrotic cell death are conserved from nematodes to humans and that intracellular acidification is an important event in the course of neurodegeneration. Thus, the physiological pathways facilitating cellular pH homeostasis are attractive, potential targets for therapeutic intervention to battle neurodegenerative disorders.

### Supplemental Data

Experimental Procedures, Table S1, and Figure S1 are available with this manuscript online at http://www.current-biology.com/cgi/content/full/15/13/1249/DC1/.

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