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# Sirtuin inhibition protects from the polyalanine muscular dystrophy protein PABPN1

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**Oculopharyngeal muscular dystrophy (OPMD) is caused by polyalanine expansion in nuclear protein PABPN1 [poly(A) binding protein nuclear 1] and characterized by muscle degeneration. Druggable modifiers of proteotoxicity in degenerative diseases, notably the longevity modulators sirtuins, may constitute useful therapeutic targets. However, the modifiers of mutant PABPN1 are unknown. Here, we report that longevity and cell metabolism modifiers modulate mutant PABPN1 toxicity in the muscle cell. Using PABPN1 nematodes that show muscle cell degeneration and abnormal motility, we found that increased dosage of the sirtuin and deacetylase *sir-2.1*/SIRT1 exacerbated muscle pathology, an effect dependent on the transcription factor *daf-16*/FoxO and fuel sensor *aak-2*/AMPK (AMP-activated protein kinase), while null mutants of *sir-2.1*, *daf-16* and *aak-2* were protective. Consistently, the Sir2 inhibitor sirtinol was protective, whereas the Sir2 and AMPK activator resveratrol was detrimental. Furthermore, rescue by sirtinol was dependent on *daf-16* and not *aak-2*, whereas aggravation by resveratrol was dependent on *aak-2* and not *daf-16*. Finally, the survival of mammalian cells expressing mutant PABPN1 was promoted by sirtinol and decreased by resveratrol. Altogether, our data identify Sir2 and AMPK inhibition as therapeutic strategies for muscle protection in OPMD, extending the value of druggable proteins in cell maintenance networks to polyalanine diseases.**

## INTRODUCTION

The myopathy OPMD (oculopharyngeal muscular dystrophy) is caused by expanded polyalanines (polyAlas) in PABPN1 [poly(A) binding protein nuclear 1] and is characterized clinically by progressive dysphagia, ptosis and proximal limb weakness after the age of 50 (1). Normal PABPN1 contains 10 Alanines (Alas), expanded to 11–17 (most frequently 13) Alas (1,2). PABPN1 may be involved in RNA polyadenylation (3), active transport of poly(A) mRNAs (4), and control of gene expression in muscles (5). Mutant PABPN1 induces nuclear inclusion (NI) formation (6,7) and may impair molecular chaperones (8,9) and members of the ubiquitin–proteasome pathway (9). However, NIs may be unrelated to pathogenicity as expanded polyAlas are dispensable for NI formation (10,11). Additionally, live microscopy has revealed that cells harboring an increased amount of soluble mutant PABPN1 are more prone to death than those with NIs (12). While RNAs and proteins may be sequestered in NIs (13,14), which may disturb cell homeostasis, the net outcome of NI formation may thus be cell protection, and soluble mutant PABPN1 may be the primary cause for cytotoxicity in OPMD (1,15). Consistently, chemical inducers (rapamycin) of macroautophagy, a biological process mostly effective against soluble proteins, are able to reduce the cytotoxicity of aggregation-prone proteins including polyalanine proteins (16).

Druggable modifiers of proteotoxicity in degenerative diseases like sirtuins may constitute useful therapeutic targets (17–19). However, the modifiers of cytotoxicity produced by mutant PABPN1 are unknown. Besides muscle-specific pathways, core modulators of stress response may be important to muscle cell survival processes (20). To investigate the genetic modulators of mutant PABPN1 toxicity in the

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muscle cell, we generated *Caenorhabditis elegans* transgenics expressing human PABPN1 with 0, 10 or 13 Alas. Using animals that stably express GFP fused to nuclear localization signals under the control of the minor myosin heavy chain gene *myo-3* (*Pmyo-3*) to direct expression in muscle cell nuclei, we engineered integrated arrays encoding PABPN1 in muscle cell nuclei using the same promoter. These animals expressed PABPN1 with 0, 10 or 13 Alas at similar levels, and the transgenic phenotypes observed comprised adult-stage abnormalities, notably motility defects and muscle cell degeneration produced by mutant PABPN1 (PABPN1-A13) and not by normal PABPN1 (PABPN1-A10) expression.

Using these animals, we show that key members in longevity and cell metabolism networks modify PABPN1-A13 cytotoxicity. The deletion of the class III protein deacetylase *sir-2.1*/SIRT1, the transcription factor *daf-16*/FoxO and the fuel sensor *aak-2*/AMPK (AMP-activated protein kinase) rescued *C. elegans* adult phenotypes, whereas increased *sir-2.1* dosage was detrimental in a *daf-16* and *aak-2* dependent manner. Additionally, the Sir2 inhibitor sirtinol protected *C. elegans* transgenics from PABPN1-A13, an effect dependent on *sir-2*.1, *daf-16* but not *aak-2*. Consistently, the Sir2 (19,21,22) and AMPK (23) activator resveratrol was detrimental through *sir-2.1*, *aak-2* but not *daf-16* activity. Sirtinol and resveratrol showed similar profiles of activity in mammalian cells overexpressing mutant PABPN1. Thus, while resveratrol may protect the Huntington disease (HD) neuron from the burden of polyglutamine (polyQ)-expanded huntingtin cytotoxicity through FoxO activity (19), this compound may use other targets like AMPK (23) which may be detrimental to OPMD muscles. Sir2 inhibition but not activation may thus attenuate mutant PABPN1 cytotoxicity by eliciting FoxO-dependent protective mechanisms, indicating that treatment with sirtuin inhibitors may ameliorate OPMD pathology. Additionally, our data suggest that AMPK inhibitors may protect OPMD muscles.

## RESULTS

### Mutant PABPN1 produces muscle pathology in adult *C. elegans*

We engineered animals expressing PABPN1 (with 0, 10 or 13 Alas) and GFP in the form of two separate proteins under the same promoter P*myo-3* as we observed GFP::PABPN1 fusion proteins produce some toxicity unrelated to polyAlas, masking the magnitude of polyAla toxicity (data not shown). *Pmyo-3* is expressed in 104 cells, including all body wall muscle cells, and a few other muscle cells (24). We generated transgenic strains that express integrated arrays encoding similar level of PABPN1 (with 0, 10 or 13 Alas). The expression of integrated arrays encoding PABPN1-A13 specifically produced muscle defects in adults. In culture, *C. elegans* moves by crawling across bacterial lawns in a highly regular sinusoidal pattern, which can be quantified and scored as the number of whole body bends per unit time (25) and the amplitude of the sinusoidal wave measured from tracks across a bacterial lawn. Compared with controls, 5-day old PABPN1-A13 animals showed uncoordinated phenotypes including decreased amplitude of the sinusoid wave (Fig. 1A). This abnormal motility phenotype was accompanied by an increased frequency of body bends in PABPN1-A13 animals, an effect not observed in PABPN1-A10 animals (Fig. 1B). These phenotypes were not due to differences in transgene RNA (Supplementary Material, Table S1) or protein (Supplementary Material, Fig. S1) expression or to differences in aging rates as all the PABPN1 strains showed the same lifespan (data not shown). These phenotypes were observed in two independent strains expressing integrated arrays encoding PABPN1-A13 (data not shown).

PABPN1 is normally found in the nucleoplasm of muscle cells, forming NIs in muscle fiber nuclei (13). To test for the localization of human PABPN1 in the transgenic *C. elegans*, we stained whole transgenic worms with a human PABPN1 antiserum. Immunostaining revealed a diffuse expression of PABPN1 with 0, 10 and 13 Alas localized to the nucleoplasm of GFP positive nuclei, with some accumulation observed for PABPN1-A13 (Fig. 1C). Weak accumulation was observed in 20–30% of cells of PABPN1-A13 animals. This was consistent with the notion that proteotoxicity in OPMD primarily involves soluble forms of mutant PABPN1 (11,12,15).

We next examined the appearance of muscle cells by scoring the number of fluorescent muscle cell nuclei. Compared with GFP animals, PABPN1-A0 and PABPN1-A10 animals were unaffected, whereas PABPN1-A13 animals showed a progressive loss of GFP signals (Fig. 1D), suggesting the occurrence of degenerative processes. To test for muscle cell degeneration, we further examined muscle cell structure by staining of actin filaments with a rhodamine–phalloidin fluoroprobe. PABPN1-A13 animals showed greater muscle cell defects and cell loss compared with the basal level showed by GFP, PABPN1-A0 and PABPN1-A10 animals (Fig. 1E).

Altogether, these data indicated that transgenic nematodes show a progressive muscular pathology produced by soluble forms of mutant PABPN1, providing a useful system to identify genetic and chemical modifiers of a toxic component difficult to manipulate *in vivo* by other means (7).

### Sodium butyrate rescues muscle pathology in nematodes and H4 hypoacetylation in mammalian cells

Besides regulating mRNA processing, human PABPN1 may control the expression of muscle-specific genes (5). In muscle cells, PABPN1 is associated with SKIP, a protein that binds to the oncoprotein SKI in a complex that regulates transcription (5). SKIP is a conserved protein (26) that interacts with members of the histone deacetylase (HDAC) complex (27). Additionally, deacetylase inhibitors may protect dystrophic muscles in MDX mice (28) and fibroblasts from spinal muscular atrophy patients (29). We thus hypothesized that mutant PABPN1 cytotoxicity may involve altered histone acetylation. To test this hypothesis, we incubated PABPN1-10 and PABPN1-A13 worms with the HDAC inhibitor sodium butyrate. Butyrate showed a strong (the maximal achievable rescue is 28%) rescue of the loss of GFP signals caused by PABPN1-A13 expression (Fig. 2A). Butyrate treatment did not affect the level of transgene expression (Supplementary Material, Table S1). Additionally, butyrate

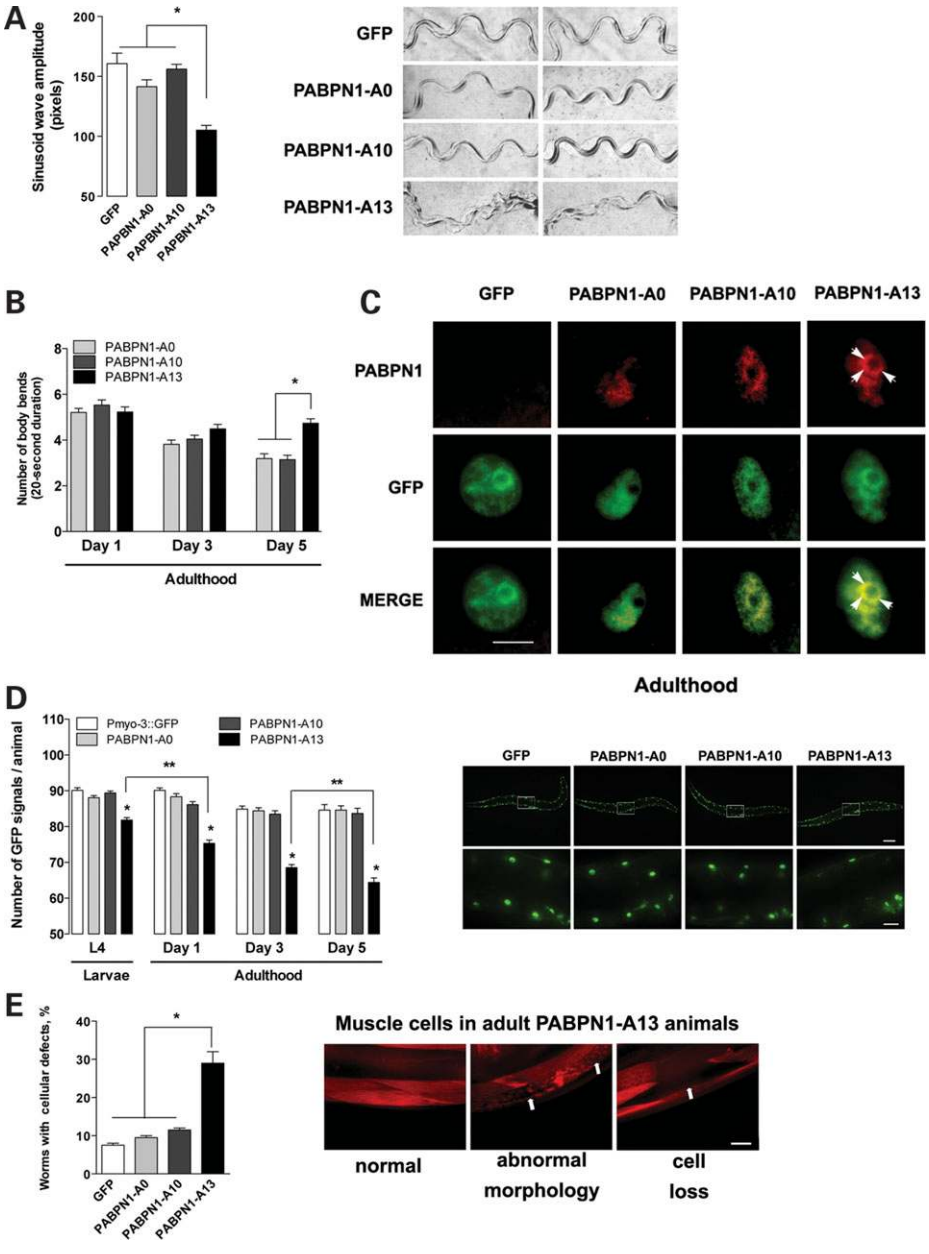


**Figure 1.** Mutant PABPN1 produces muscle pathology in adult *C. elegans*. (**A**) Abnormal motility induced by mutant PABPN1 (PABPN1-A13) is shown as the amplitude of the sinusoid wave (mean ± SEM). $^{*}P < 0.001$ compared with each of the controls. The right panel shows representative images of the sinusoid waves. (**B**) Abnormal motility induced by PABPN1-A13 is shown as the number of body bends in 20-s periods (mean ± SEM). $^{*}P < 0.001$ compared with PABPN1-A0 and PABPN1-A10. (**C**) Expression of PABPN1 in muscle cell nuclei. Staining of whole worms with a human PABPN1 antibody (in red) revealed a diffuse expression of the protein in GFP-positive nuclei of PABPN1-A0, PABPN1-A10 and PABPN1-A13, with some weak accumulation (white arrows) observed for PABPN1-A13. Scale bar is 5 μm. (**D**) Age-dependent loss of GFP signals induced by PABPN1-A13. The left panel shows the number of GFP signals/animal (mean ± SEM). $^{*}P < 0.001$ compared with PABPN1-A10. $^{**}P < 0.001$ compared to as indicated. The right panel shows GFP signals in representative young adults (upper panels: magnification is ×10 and scale bar 100 μm) and corresponding body wall muscle nuclei (lower panels: magnification is ×63 and scale bar 20 μm). (**E**) Muscle cell degeneration induced by PABPN1-A13. The left panel shows the percentage of worms with abnormal or missing muscle cells as visualized by staining of actin filaments. $^{*}P < 0.001$ compared to each of the controls. The right panel shows staining of representative muscular cells in PABPN1-A13 animals (scale bar is 20 μm).

rescued the abnormal mobility caused by PABPN1-A13 expression with no effect in PABPN1-A10 animals (Fig. 2B).

To test whether mutant PABPN1 may affect histone acetylation in mammalian cells, we analysed acetylation levels of histones H3 and H4 in COS-7 cells transfected with PABPN1 constructs. The expression of mutant PABPN1 with 17 Alas reduced acetylation of H4, but not H3, compared with normal PABPN1 with 10 Alas (Fig. 2C). When these cells were treated with butyrate, levels of H4 and H3 acetylation in cells expressing normal or mutant PABPN1 were increased (Fig. 2C). While acetylation levels were altered, total histone levels, determined by silver staining of proteins

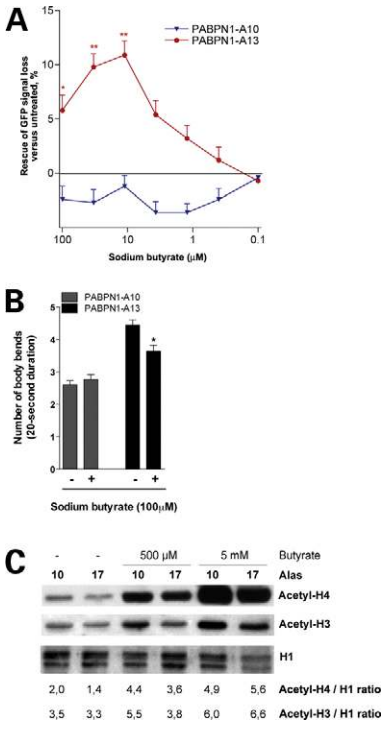


**Figure 2.** Sodium butyrate rescues muscle pathology in nematodes and H4 hypoacetylation in mammalian cells. (**A**) Sodium butyrate rescues the loss of GFP signals induced by mutant PABPN1 at day 1 in *C. elegans*. Protection is shown as percent rescue versus untreated controls. The maximal achievable rescue in PAPBN1-A13 animals is 28% and calculated as ((A13-A10)/A10*100). $^{**}P < 0.001$ and $^{*}P < 0.05$ compared with untreated controls. (**B**) Sodium butyrate rescues the abnormal frequency of body bends caused by PABPN1-A13 expression. $^{*}P < 0.001$ compared with untreated controls. (**C**) A reduction in acetylation of histone H4 is induced by mutant PABPN1 in mammalian cell culture. COS-7 cells expressing mutant PABPN1 with 17 Alas showed a $29.7 \pm 0.4\%$ ($P < 0.01$ compared with PABPN1-A10) reduction in the level of acetylation of histone H4 compared to cells expressing normal PABPN1 with 10 Alas. The level of acetyl H3 was not significantly influenced by PABPN1-A17. Incubation with 500 μM and 5 mM sodium butyrate increased the level of acetylation of histone H4 in cells expressing normal or mutant PABPN1, an effect observed at lower intensity for histone H3 compared with histone H4. Equivalent levels of histones are shown with anti-histone H1.

and immunostaining of total H1 histones, were unchanged by expressing PABPN1 proteins (Fig. 2C). Thus, mutant PABPN1 expression causes a reduction in H4 acetylation that is reversed by sodium butyrate, providing insight into the mechanisms for improvement of muscular dystrophy by HDAC inhibitors (28).

## Mutant PABPN1 cytoxicity is modulated by *sir-2.1*, *daf-16* and *aak-2*

Having shown *C. elegans* allows the effect of deacetylase modulators to be detected on the muscle pathology produced by mutant PABPN1, we investigated more specifically whether class III deacetylases, also referred to as sirtuins (Sir2), may be involved in the modulation of mutant PABPN1 cytotoxicity. Sir2 is a major and conserved modulator of longevity (30). Sir2 activation may protect against neurodegenerative disease-associated proteins, and Sir2 protection against expanded polyQs may require the transcription factor *daf-16*/FoxO (19,31). Unlike what we observed for the expression of mutant polyQs in neurons (19), increased *sir-2.1*/SIRT1 dosage strongly enhanced the loss of GFP signals in PABPN1-A13 animals, whereas weak enhancement was observed in GFP, PABPN1-A0 and PABPN1-A10 strains (Fig. 3A). Consistently, a null mutation of *sir-2.1* or gene knock-down by *sir-2.1* RNAi rescued the loss of GFP signals in PABPN1-A13 animals. The gene knock-down by *sir-2.1* RNAi similarly enhanced the loss of GFP signals in control strains, indicating the rescue of GFP signal loss is specific to PABPN1-A13 animals (Fig. 3A). The reduction of SIR-2.1 expression by *sir-2.1* RNAi was verified by western blotting (Supplementary Material, Fig. S2). Next, we investigated the effects of *daf-16*/FoxO. *daf-16* genetically interacts with *sir.2-1* in modulating longevity (32) and protection against the effects of neurodegenerative disease-associated polypeptides (19,33). A null mutation of *daf-16* similarly enhanced the loss of GFP signals in GFP, PABPN1-A0 and PABPN1-A10 strains, whereas it rescued this phenotype in PABPN1-A13 animals (Fig. 3A), suggesting that *daf-16* is able to sustain normal muscle homeostasis and promote mutant PABPN1 toxicity. Additionally, we investigated the effects of *aak-2*/AMPK. The fuel sensor AMPK may participate in the modulation of longevity (34), may be used by the neuroprotective compound resveratrol for activity (21,23) and phosphorylate FoxO proteins (35). This suggests that AMPK may be a modulator of proteotoxicity in degenerative diseases. Consistent with this possibility, a null mutation of *aak-2* rescued the loss of GFP signals in PABPN1-A13 animals with no effect in control strains (Fig. 3A), indicating normal *aak-2* promotes mutant PABPN1 cytotoxicity. Finally, we observed that the enhancement of GFP signal loss by increased *sir-2.1* dosage in PABPN1-A13 animals was reversed back to rescue by a null mutation of *daf-16* or *aak-2* (Fig. 3A). The effects of genetic manipulations of *sir-2.1*, *daf-16* and *aak-2* were unrelated to changes in transgene expression (Supplementary Material, Table S1). Thus, *sir-2.1* genetically interacts with *daf-16* and *aak-2* in promoting the muscle nuclei pathology produced by PABPN1-A13, with the inhibition of these three genes being beneficial. In regard to behavior, *sir-2.1*, *daf-16* and *aak-2* mutants strongly rescued the abnormal motility caused by PABPN1-A13 expression with no effect in control strains as inferred from number of body bends (Fig. 3B) and sinusoid wave amplitudes (Fig. 3C). We could not examine the contribution of increased *sir-2.1* dosage on these behavioral phenotypes since the *sir-2.1* transgene is linked with the dominant *rol-6* marker and these worms cannot move in a sinusoidal pattern. Together, these data suggested that *sir-2.1*, *daf-16* and *aak-2* cooperate to modify the muscular pathology caused by mutant PABPN1, and strategies to inhibit the druggable proteins Sir2 and AMPK may be protective against OPMD pathology.

## Sirtinol and resveratrol show similar profiles of activity in *C. elegans* and mammalian cells expressing mutant PABPN1

Resveratrol, a plant polyphenol, likely has several intracellular targets (36) and is able to activate SIRT1 (37) and AMPK (23). Resveratrol is protective in models of neurodegenerative

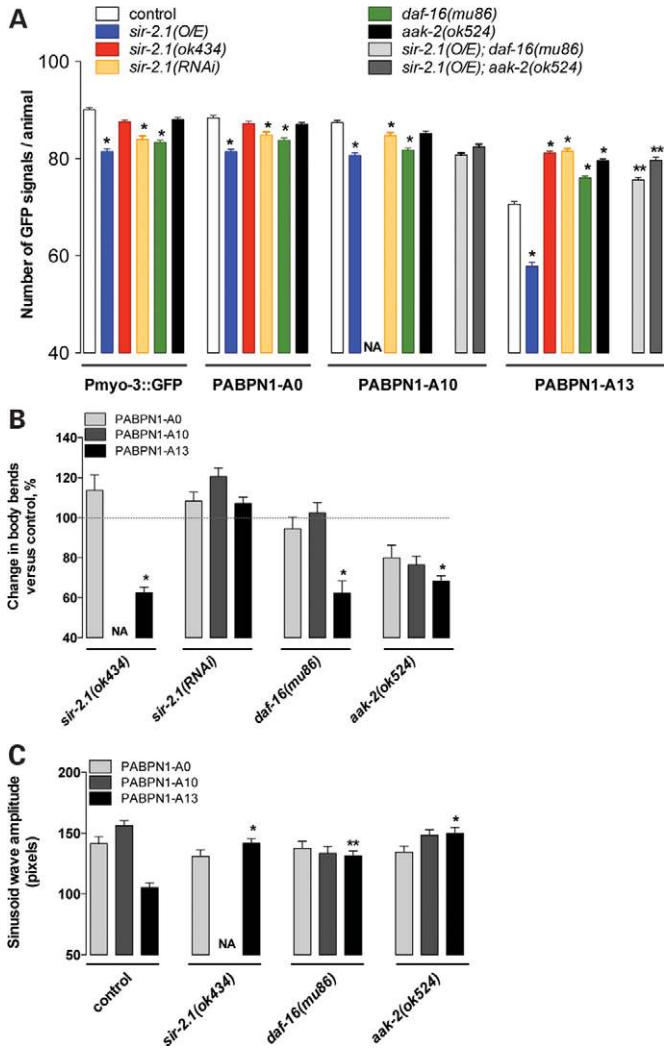


**Figure 3.** Effects of genetic manipulations of *sir-2.1*/SIRT1, *daf-16*/FoxO and *aak–2*/AMPK on mutant PABPN1 cytoxicity. (**A**) The loss of GFP signals induced by PABPN1-A13 in muscle cell nuclei was strongly enhanced by increased *sir-2.1*dosage (*O/E*) and inhibited by (i) a null mutation (*ok434*) in or gene knock-down (using RNAi; see decreased SIR-2.1 expression in Supplementary Material, Fig. S2) of *sir-2.1*, (ii) a null mutation (*mu86*) in *daf-16*/FOXO and (iii) a null mutation (*ok524*) in *aak-2*/AMPK. The PABPN1-A10;*sir-2.1*(*ok434*) animals are not available (NA) as genetic engineering was repeatedly unsuccessful, likely because the transgenic array is integrated near the *sir-2.1* locus. The *sir-2.1*(*O/E*) and *daf-16*(*mu86*) mutations and the *sir-2.1* RNAi weakly enhanced loss of GFP signals in GFP, PABPN1-A0 and PABPN1-A10 animals in a non specific manner. The enhancement of GFP signal loss by increased *sir-2.1* dosage (*O/E*) in PABPN1-A13 animals was reversed by the null mutations *mu86* in *daf-16*/FOXO and *ok524* in *aak-2*/AMPK, with no effect on the loss of GFP signals produced by increased *sir-2.1* dosage in control PABPN1-A10 animals. $^{*}P < 0.001$ compared to control transgenics. $^{**}P < 0.001$ compared to transgenics with increased *sir-2.1* dosage (*O/E*). (**B**) A null mutation of *sir-2.1*(*ok434*), *daf-16*(*mu86*) and *aak-2*(*ok524*) strongly rescued the abnormal frequency of body bends caused by PABPN1-A13 expression. The gene knock-down (RNAi) of *sir-2.1* showed no effect. Changes in body bends are calculated as ((test/control)*100). The dotted line indicates the baseline level. $^{*}P < 0.001$ compared to control transgenics. (**C**) A null mutation of *sir-2.1*(*ok434*), *daf-16*(*mu86*) and *aak-2*(*ok524*) rescued the abnormal sinusoid wave amplitude caused by PABPN1-A13 expression. $^{*}P < 0.001$ and $^{**}P < 0.05$ compared with PABPN1-A13.

diseases (19,38), metabolic disturbance (21,22) and age-decay phenotypes in vertebrates (39). Sirtinol is a synthetic Sir2 inhibitor (37) whose impact on disease cell survival is not characterized. We hypothesized these two compounds may show opposite effects in modulating the muscular pathology in PABPN1-A13 *C. elegans* transgenics. Treating PABPN1-A13 animals with sirtinol resulted in the rescue of GFP signal loss, an effect dependent on *sir-2.1*, *daf-16* but not *aak-2* (Fig. 4A). Sirtinol also rescued abnormal motility in PABPN1-A13 animals with no effect detected in PABPN1-A10 animals, an activity dependent on *sir-2.1* and *daf-16* (Fig. 4B). The effect of *aak-2* could not be tested as the null mutation of *aak-2* completely rescued the abnormal motility phenotype. Splitomicin, another Sir2 inhibitor (40), also rescued the loss of GFP signals and abnormal motility produced by PABPN1-A13 expression, with no effect in controls (Supplementary Material, Fig. S3). Sirtinol and splitomicin did not affect the level of transgene expression (Supplementary Material, Table S1). In contrast, treatment with resveratrol enhanced the loss of GFP signals in PABPN1-A13 animals, with no effect in PABPN1-A10 animals (Fig. 4C). Resveratrol did not affect the level of transgene expression (Supplementary Material, Table S1). While the enhancement of GFP signal loss by resveratrol was mediated by *sir-2.1* and *aak-2*, it was independent of *daf-16* (Fig. 4C). Resveratrol also showed a *daf-16* independent and *sir-2.1* and *aak-2* dependent activity for the enhancement of abnormal motility produced by PABPN1-A13 expression (Fig. 4D). Together, these data indicated that while sirtinol and resveratrol both require Sir2 for activity, they may use different mechanisms to modulate cell survival, namely FoxO-dependent mechanisms for muscle cell protection by sirtinol, and AMPK-dependent mechanism for aggravation of muscle cell decline by resveratrol.

Next, we used live microscopy to examine the effects of sirtinol and resveratrol in mammalian cells, here COS-7 cells overexpressing GFP–PABPN1 fusion proteins with 0, 10 or 17 Alas. In this model, mutant PABPN1 expression induces greater cell death from day 2 to 6 compared with PABPN1-A10 expression (12). At day 5, sirtinol strongly enhanced cell survival, whereas resveratrol slightly decreased cell survival in mutant PABPN1 cells, the two compounds showing no effects in cells overexpressing GFP–PABPN1 with 0 and 10 Alas (Fig. 4E). Sirtinol or resveratrol treatment did not affect the level of protein expression (Supplementary Material, Fig. S4). Thus, the profiles of activity of sirtinol and resveratrol on mutant PABPN1 cytotoxicity in mammalian cells and transgenic nematodes were similar.

Altogether, these data indicated the chemical inhibition of Sir2 may protect against muscular dystrophy in OPMD through FoxO-dependent mechanisms, whereas compounds able to activate Sir2 and AMPK like resveratrol may be detrimental through mechanisms that involve AMPK.

## DISCUSSION

Our data indicate that Sir2, FoxO and AMPK cooperate in the modulation of mutant PABPN1 toxicity and define the

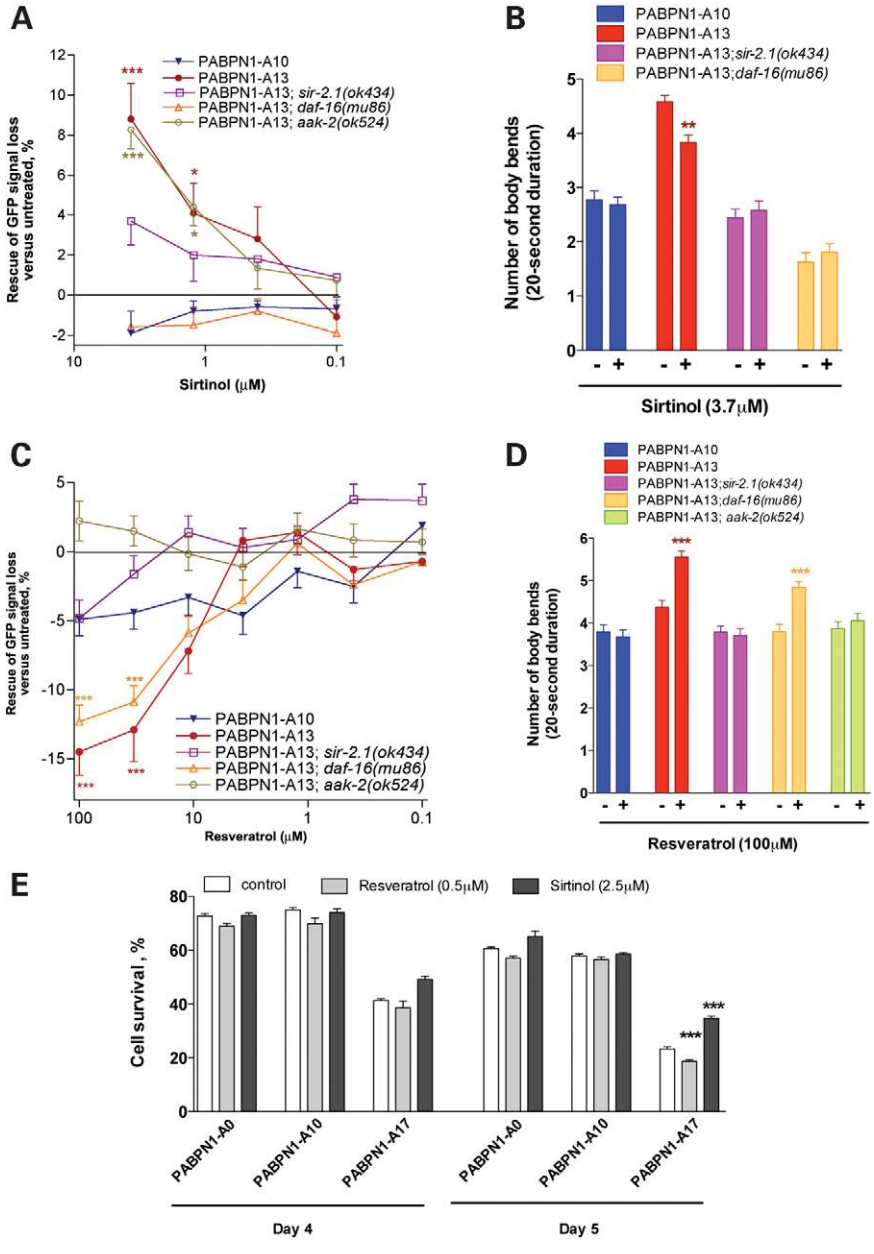


**Figure 4.** Sirtinol and resveratrol show similar profiles of activity in *C. elegans* and mammalian cells expressing mutant PABPN1. (**A**) Sirtinol protected against the loss of GFP signal in muscle nuclei in *C. elegans*, an effect lost in the presence of a null mutation of *sir-2.1*(*ok434*), *daf-16*(*mu86*) but not *aak-2*(*ok524*). The maximal achievable rescue in PAPBN1-A13 animals is 28% and calculated as ((treated − untreated)/untreated*100). $^{***}P < 0.001$ and $^{*}P < 0.05$ compared with untreated controls. (**B**) Sirtinol rescued the abnormal frequency of body bends produced by PABPN1-A13 at day 5 of adulthood in a *sir*-2.*1* and *daf-16* dependent manner. $^{**}P < 0.01$ compared with untreated controls. (**C**) Resveratrol enhanced the loss of GFP signals in *C. elegans*, an effect lost in the presence of a null mutation of *sir-2.1*(*ok434*), *aak-2*(*ok524*) and not *daf-16*(*mu86*). A statistically-significant negative value for rescue means aggravation of the phenotype. $^{***}P < 0.001$ compared with untreated controls. (**D**) Resveratrol enhanced the abnormal frequency of body bends produced by PABPN1-A13, an effect dependent on *sir-2.1*, *aak-2* and not *daf-16*. $^{***}P < 0.001$ compared with untreated controls. (**E**) Resveratrol reduced and sirtinol rescued the survival of COS-7 cells expressing mutant PABPN1 fused to GFP at day 5 with no effect observed at day 1, 2, 3 and 4. $^{***}P < 0.001$ compared with untreated cells.

inhibition of Sir2 and AMPK as new therapeutic strategies for muscle protection in OPMD. The transgenic nematodes described herein are indicative of cytotoxicity primarily produced by soluble mutant PABPN1. Several studies have suggested that soluble forms of disease proteins and polypeptides like mutant huntingtin (41), Aß (a cleavage product of the amyloid precursor protein) (18), mutant ataxin-1 (42) and mutant PABPN1 (12) may be major cytotoxic components. These animals thus provide a unique system for the genetic and chemical manipulation of a toxic component difficult to isolate and study *in vivo* by other means (7). Transgenic *C. elegans* do not explain the peculiar muscle specificity of OPMD as the expression of PABPN1, a ubiquituously-expressed protein in humans, was directed to worm muscles. Although transgenic phenotypes were produced by PABPN1 overexpression, overexpression systems have been widely

instructive to study the gain-of-function(s) produced by degenerative disease proteins, and our data indicate that PABPN1 nematodes are useful for defining disease targets. The rescue of muscle pathology by the deacetylase inhibitor sodium butyrate in PABPN1 nematodes is consistent with the notion that increased levels of histone acetylation may protect the dystrophic muscle (28). Additionally, the nematodes described herein allowed us to identify key longevity and cell metabolism modulators as modifiers of mutant PABPN1 cytotoxicity. Proteins at the interface between organismal longevity and cell survival like Sir2/SIRT1 have been associated with neuronal cell protection (17,19,31,43). These studies have emphasized neuroprotection by Sir2/SIRT1 activation. In contrast, our data indicate that Sir2 inhibition protects against OPMD muscle pathology, whereas Sir2 activation is detrimental. Interestingly, sirtuin 2 (SIRT2) inhibition may rescue the cytotoxicity produced by α-synuclein, a protein associated to Parkinson's disease (PD) pathogenesis (44). Thus, while sirtuins may be disease targets for both neurodegenerative diseases (HD, PD) and muscular dystrophies (OPMD), the way to achieve cell protection against a specific pathological condition may best rely on either sirtuin activation or inhibition. Regarding homopolymer expansion, the findings reported herein and our previous data (19) indicate that cell protection by manipulating Sir2 involves *daf-16*/FoxO, which is in agreement to what has been shown for the modulation of longevity (32). We speculate the modulation of DAF-16 acetylation and activity by SIR2 may have different outcomes depending on the cell type and stress condition encountered (45). Together with previous reports indicating that an excess of FOXO activity may promote autophagy in muscles (46,47) and muscle atrophy (48) and may suppress muscle differentiation (49), our genetic data indicate that FoxO is a key modulator of muscle cell homeostasis in normal and pathological conditions, and additional studies will be needed to identify the mechanisms underlying FoxO-dependent protection of the diseased cell.

Our data indicate that *aak-2*/AMPK may contribute to mutant PABPN1 toxicity and is required for enhancement of mutant PABPN1 toxicity by increased Sir2 dosage and by resveratrol. Besides activity that requires SIR2 (19), resveratrol may behave as an AMPK activator as it increases AMPK levels in mice on a high-calorie diet (21) and stimulates AMPK in cultured neurons (23). Additionally, AMPK may regulate FoxO proteins (35). Together with these reports, our observation suggests that AMPK and SIR2 may similarly influence cell processes, their inhibition resulting in the protection of diseased muscles. Interestingly, we observed that a null mutation in *aak-2* enhanced touch receptor neuron dysfunction in *C. elegans* transgenics expressing expanded polyQs (A. Parker and C. Néri, unpublished data), which is similar to the effect of a null mutation in *sir-2.1* in these animals. This raises the possibility that the manipulation of Sir2 and AMPK activity may show opposite effects in the polyQ neurons compared with the polyAla muscles, their inactivation being protective in OPMD muscles and detrimental in HD neurons.

Consistent with the genetic data, the Sir2 inhibitors sirtinol and splitomicin promoted and the Sir2 activator resveratrol reduced muscle protection in PABPN1 nematodes. Rescue of muscle pathology by sirtinol was dependent on *sir-2.1* and *daf-16* but not *aak-2*. This suggests FoxO is involved in the Sir2-dependent chemical protection of diseased muscles, similarly to what has been observed for the protection of diseased neurons (19). The enhancement of muscle pathology by resveratrol was dependent on *sir-2.1*, *aak-2* and not *daf-16*. This contrasts with our genetic data indicating that the enhancement of mutant PABPN1 cytotoxicity by increased *sir-2.1* dosage is dependent on both *aak-2* and *daf-16*, and may be due to the fact that, besides Sir2/SIRT1, resveratrol activity may involve other targets including AMPK (23). Nonetheless, the effects of *aak-2* loss-of-function used alone or in combination to either increased *sir-2.1* dosage or resveratrol treatment indicate that AMPK inhibitors may ameliorate OPMD pathology.

Consistent with their profile of activity in PABPN1 nematodes, sirtinol promoted and resveratrol reduced the survival of mammalian cells overexpressing mutant polyAlas in the context of GFP–PABPN1 fusion proteins. Together with previous findings on resveratrol activity in polyQ nematode neurons and striatal neurons from huntingtin knock-in mice (19), these results further indicate that Sir2 modulators show similar profiles of activity in *C. elegans* and mammalian cells expressing a mutant disease protein.

Resveratrol has been reported to show several beneficial effects among which the improvement of motor and muscle function in mice on a high-calorie diet (21,22). In contrast, our study indicates that resveratrol aggravates mutant PABPN1 toxicity in the nematode muscle and in mammal cells. This suggests that the effect of resveratrol is context-dependent, increasing the resistance to muscle fatigue while enhancing the susceptibility to degeneration of the dystrophic muscle. The PABPN1 protein may have a role in muscle gene expression (5) and when mutated may modify the beneficial effect of resveratrol on the control of energy metabolism in the muscle (21,22), leading to aggravation of muscle cell decline. In contrast, the Sir2 inhibitor sirtinol protects the muscle cell from mutant PABPN1 toxicity, which, together with the evidence for synthetic SIRT2 inhibitors to protect against neurodegenerative disease proteins like alpha-synuclein (44) suggests that sirtuin inhibition might have general therapeutic potential.

In conclusion, our genetic data indicate that Sir2 inhibition may protect OPMD muscles while Sir2 activation may be detrimental. Our pharmacological data indicate that muscle protection by sirtinol involves FoxO and not AMPK, whereas muscle pathology aggravation by resveratrol may involve AMPK and not FoxO, both compounds being dependent on Sir2 for activity and able to modulate mutant PABPN1 toxicity in mammalian cells. Additional experiments will be needed to establish the mechanisms underlying the modulation of PABPN1 cytotoxicity by *daf-16*/FoxO and *aak-2*/AMPK in response to manipulation of Sir2. Finally, our data emphasize Sir2 inhibitors and AMPK inhibitors as potential therapeutics for OPMD and perhaps other muscular dystrophies and polyAla diseases.

## MATERIALS AND METHODS

### *C. elegans*

*Reporter constructs*. Using PABPN1 sequence with 10 and 13 Alas (Centre for Research in Neurosciences, McGill

University, Montreal, Canada), we constructed *Pmyo-3*::PABPN1 (with 0, 10 and 13 Alas) GFP fusions conducted through PCR and subcloning into the *Eco*RI sites of the *myo-3::GFP* vector pPD118.20 (A. Fire, Washington University, St Louis, MO). Primers used for amplification of 10 and 13 Alas were: 5′–3′ (pabF) CGGGAATTCATGGCGGCGGC GGCGGCGGCG, and (pabR) CGGGAATTCTTAGTAAG GGGAATACCATGATGTTGTCGC. We used an alternate forward primer to amplify PABPN1 with 0 Ala (pab0F) CGG GAATTCATGGG-GGCTGCGG). We constructed *Pmyo-3*::PABPN1 (with 0, 10 and 13 Alas) conducted through removal of GFP with digestion using *Xba*I and *Btg*ZI enzymes. All constructs were sequenced to verify integrity.

*Genetic manipulations*. Nematode strains were received from the *Caenorhabditis elegans* Genetics Center CGC (St Paul, MN) unless indicated otherwise and were maintained following standard methods. Transgenic strains expressing integrated arrays and similar levels of transgene expression were generated from strains expressing extrachromosomal arrays by standard techniques. Details for the construction, characterization and use in genetic crosses of nematode transgenics are available in the Supplementary Material section.

*Motility assays*. 1–5-day-old adult hermaphrodites were laid on confluent OP-50-1. The animals were then scored immediately for whole body bends for a duration of 20 s as previously described (25), or photos of the tracks were taken using a 2.5× objective on a Zeiss microscope (Axioplan Imaging II) and their amplitudes measured using MetaView software (Molecular Devices). A total of 100 animals were scored per experiment and three independent assays performed. Percent change of the body bends phenotype was calculated as ((test/control)*100). ANOVA tests and Tukey's Multiple Comparison Tests were used for statistics.

*Lifespan assays*. Fifty synchronized L4 larvae were laid on OP-50-1 bacteria plates (10 animals/plate) at 20°C and transferred to new bacterial plates every other day. Worms were scored every day for normal motility, abnormal motility, paralysis and death. Three independent assays were performed. The Logrank test (Prism software) was used for the statistical analysis of survival curves.

*Analysis of muscle cells*. Muscle cells in transgenic hermaphrodites were examined for loss of nuclei through counting all GFP positive cells. A total of 100 worms were scored per strain and three independent assays performed. Muscle cells in transgenic hermaphrodites were examined for PABPN1 expression through fluorescent microscopy. Whole animals (analysis of a total of 60–70 worms per strain in three independent experiments) were permeabilized and stained with the PABPN1 antiserum VEADa-14 (Centre de Recherche du CHUM, Montreal; 1:50), and fluorescent microscopy was conducted on a Zeiss microscope (Axioplan Imaging II). Observations of the structure of body wall muscles in adult transgenics after Phalloidin–Rhodamin (FluoProbes) staining were performed as previously described (50). One hundred worms were scored per strain and three independent assays performed. ANOVA tests and Tukey's Multiple Comparison Test were used for statistics.

*Drug assays*. Synchronized populations of L1 larvae expressing GFP and PABPN1 with 10 or 13 Alas were obtained by hypochlorite extraction. To test for muscle nuclei loss, animals were grown in 96-well microtiter plates in 50 μl liquid culture medium with sodium butyrate, resveratrol, sirtinol or splitomicin (Sigma) for 65 h at 20°C, transferred to agarose pads on slides and scored for cell nuclei loss at 10–63× using a Zeiss fluorescence microscope (Axioplan Imaging II). Sixty worms were scored per point and three independent assays performed. Percent rescue was calculated as ((test−control)/control*100). To test for motility, the synchronized L1 larvae were grown on NGM plates containing drugs until they became 1–5-day-old adults. Animals were transferred to new plates every other day to keep them separate from progeny, then transferred to confluent OP-50-1 bacteria test plates and assessed for the number of whole body bends as described earliar (see Motility assays).

*Western blot analysis*. To test for PABPN1 expression levels, proteins from L4 larvae were extracted as described (51), separated by SDS/PAGE, analysed by western blotting with a human PABPN1 antiserum (52) or actin antibody (MP Biomedicals; 1:6,000). To test for SIRT1 expression, worm proteins were prepared as previously described (53). Protein extracts were probed using a SIRT1 antibody (Upstate; 1:3000) and actin antibody (MP Biomedicals; 1:6,000). Western blots were revealed using the western blot chemiluminescence reagent Plus kit (GE Healthcare).

## Mammalian cells

*Plasmid constructs*. The cDNAs encoding PABPN1 with 10 or 17 Alas (Centre for Research in Neurosciences) were cloned into the pEGFP-C2 vector (Clontech, Palo Alto, CA), resulting in a fusion of GFP to the N-termini of PABPN1 proteins. The QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate the PABPN1-A0 construct. All constructs were verified by DNA sequencing.

*Cell culture and transfection*. Twenty-four hours before transfection, COS-7 cells were seeded in Dulbeco's modified Eagle's medium (Gibco BRL, MD) containing 10% fetal calf serum (Gibco BRL) at a concentration of $2 \times 10^5$ cells per well in 12-well plates suited for the automated live microscopy. The cells were transfected with plasmid DNA (1 μg) pre-complexed with the Plus reagent and diluted in Lipofectamine reagent (Gibco BRL) according to the manufacturer's instructions. To check the protein expression before and after the drug treatment, parallel plates were prepared for western blotting.

*Histone acetylation analysis*. COS-7 cells transfected with normal (10 Alas) and mutant (17 Alas) PABPN1 cDNAs were plated for 48 h and treated for the last 24 h with 500 μM or 5 mM sodium butyrate. Controls included non-butyrate-treated cells. Cells were lysed in 10 mM Tris, 50 mM sodium bisulfite, 1% triton X-100, 10 mM magnesium

chloride, 8.6% sucrose, pH 6.5 and a cocktail of proteases inhibitors (Roche, Diagnostics GmbH, Mannhein, Germany) for 10 min. Proteins were extracted under acidic conditions as described (54). Ten micrograms of whole cell extract were analysed by western blot. Anti-acetylated histone H3 lysine 14 and anti-acetylated histone H4 lysines 5, 8, 12, 16 and anti-histone H1 (all from Upstate Biotechnology) were used to determine levels of acetylated histones H3 and H4 relative to levels of total histones H1. Signals were quantified using NIH IMAGE 1.62 and *t*-tests used for statistical analysis.

*Drug treatment*. Resveratrol and sirtinol (Sigma) were dissolved in DMSO. In the initial cell treatment experiments, we performed dose–response experiments, using non-transfected cells, to define the most appropriate doses to be used for the drugs. For drug testing, 0.5 μM resveratrol and 2.5 μM sirtinol were the appropriate doses, as they did not cause cell toxicity for the period of experiments (5 days), and therefore they were used throughout the study. The drugs or vehicle as a control were applied on transfected cells 24 h post-transfection. Cells (in the 12-well plate) were then incubated for 24 h before analysis using a Leica automated microscope stage.

*Assessment of cell survival*. Cell survival was assessed using live microscopy as previously described (12). Details are available in the Supplementary Material.

*Western blotting*. Cells were harvested at different time points post-transfection and proteins extracted in lysis buffer. Equal amounts of protein were analyzed on 12% SDS–PAGE and transferred to a nitrocellulose membrane. The membranes were probed with a GFP antibody (Clontech, 1:2000) and revealed using the western blot chemiluminescence reagent Plus kit (NEN Life Science Products). Parallel samples were probed with an actin antibody (Chemicon) to verify equal loading of lysates.

## SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

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