Drosophila as a potential model to ameliorate mutant Huntington-mediated cardiac amyloidosis

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Several human diseases, including Huntington's disease (HD), are associated with the expression of mutated, misfolded, and aggregation-prone amyloid proteins. Cardiac disease is the second leading cause of death in HD, which has been mainly studied as a neurodegenerative disease that is caused by expanded polyglutamine repeats in the huntingtin protein. Since the mechanistic basis of mutant HD-induced cardiomyopathy is unknown, we established a Drosophila heart model that exhibited amyloid aggregate-induced oxidative stress, resulting in myofibrillar disorganization and physiological defects upon expression of HD-causing PolyQ expression in cardiomyocytes. Using powerful Drosophila genetic techniques, we suppressed mutant HD-induced cardiomyopathy by modulating pathways associated with folding defects and oxidative stress. In this addendum, we describe additional potential molecular players that might be associated with HD cardiac amyloidosis. Drosophila, with its high degree of conservation to the human genome and many techniques to manipulate its gene expression, will be an excellent model for the suppression of cardiac amyloidosis linked to other polyglutamine expansion repeat disorders.

Commentary

HD is a rare neurodegenerative disorder resulting from expanded poly-glutamine (PolyQ) repeats in the huntingtin (HTT) protein, leading to HTT misfolding and the formation of pathogenic aggregates in neuronal tissue.[1] Several epidemiological studies have suggested a link between HD and cardiac amyloidosis, a group of diseases characterized by protein misfolding and the accumulation of aggregates in the heart.[2-4] Heart failure is the second leading cause of death in HD patients, accounting for over 30% of HD deaths compared to 2% of age-matched controls.[2-6] However, the mechanisms of PolyQ-induced cardiac defects are unknown and currently, no treatments exist to ameliorate heart failure due to HD. Since HTT is expressed in cardiac muscle and PolyQ aggregates have been reported in non-CNS tissues,[3] we postulated that cardiac expression of mutant PolyQ leads to pathogenic HTT aggregate formation in cardiac tissue and subsequent heart dysfunction. To study this, we generated a model for cardiac amyloidosis caused by expanded polyglutamine repeats in HTT using the genetically tractable *Drosophila melanogaster* system.[7]

Drosophila has long been used as a model for studying congenital heart diseases, since many of their genes controlling heart function and development are conserved (reviewed by Bier and Bodmer).[8] More recently the physiological basis of cardiac dysfunction in adult Drosophila due to aging and disease has been characterized using semi-intact heart preparations in conjunction with high speed digital imaging,[9] as well as *in vivo* by optical coherence tomography.[10] For example, cardiac expression of mutant HTT using the upstream activating sequence (UAS)-Gal4 system in conjunction with the Hand driver has allowed us to determine the deleterious effects of mutant PolyQ on cardiac structure and function.[7] Thus, Drosophila is a useful model for studying functional abnormalities associated with HD cardiac pathogenesis.

Mutant PolyQ Can Aggregate in the Heart, Causing Cardiac Dysfunction and Myofibrillar Degradation

In our recent publication,[7] we sought to explore the cardiac defects associated with expression of aggregation-prone mutant PolyQ, which is known to cause HD at PolyQ repeat lengths greater than 35. Although epidemiological studies have suggested cardiac defects in HD patients,[2-5] it remains unclear if these defects result from primary (autonomous) defects in the cardiomyocytes or if expression of mutant HTT in neurons contributes to secondary (non-autonomous) cardiac pathogenesis. Expression of the aggregation prone exon-1 Htt fragment has been used to explore the mechanisms of HD in other tissues, including skeletal muscles.[11-13] In our Drosophila model, we observed severe cardiac dilation, contractile defects, and cardiac arrhythmias in a PolyQ length-dependent manner due to cardiac expression of mutant PolyQ.[7] It is unknown whether these defects are due to cell autonomous expression of mutant PolyQ since the Hand gene is expressed in pericardial nephrocytes and lymph gland haematopoietic progenitors in addition to cardioblasts.[14] Mutant PolyQ hearts also exhibited structural abnormalities, including myofibrillar disarray and loss of actin and myosin-containing myofibrils. These cardiac defects were accompanied by reductions in the lifespan of flies expressing mutant PolyQ. Interestingly, green fluorescent protein (GFP)-tagged mutant PolyQ protein aggregates were detected in the form of GFP+ puncta in hearts expressing pathogenic lengths of PolyQ repeats compared to non-pathogenic PolyQ-25. Thus, cardiac expression of toxic PolyQ is sufficient to cause both functional and structural cardiac defects and may be associated with aggregation of mutant PolyQ. In support of these findings, a mouse model for mutant PolyQ resulted in heart failure by 12 weeks of age and the formation of aggregates that were immunoreactive for anti-amyloid in the heart.[15] Unlike our Drosophila model,[7] R6/2 mice expressing mutant PolyQ did not exhibit cardiac remodeling or cell death.[16] It is possible that the GFP tag used in our model may have increased the susceptibility for aggregation of mutant PolyQ and resulted in more severe defects, including cardiac dilation and non-contractile regions of the heart. However, aggregates were not observed in control hearts expressing GFP-tagged non-pathogenic PolyQ-25, suggesting that the GFP tag alone does not cause aggregation of HTT. It remains unclear if aggregation of PolyQ was directly causative of cardiac dysfunction in mice[16] and our Drosophila model.[7] However, amyloid formation has been observed in other cardiac amyloid models, including a murine model for desmin related cardiomyopathy (mutant alphaB crystallin R120G).[15] Based upon these findings, we predict that pathogenic aggregates accumulate in cardiac tissue of HD patients and contribute to cardiomyopathy. It remains unknown if the aggregates observed in our model contain amyloid deposits.[7] Novel therapeutic strategies to reduce protein aggregation may prove useful in clinical settings to treat PolyQ-induced cardiac amyloidosis.

Aggregation of mutant PolyQ is suspected to play a major role in HD cardiac pathogenesis by interfering with several cellular pathways, including the unfolded protein response (UPR) and the oxidative stress response.[17-19] Mouse models have shown that expression of mutant HTT or expression of pre-amyloid oligomers is sufficient to cause cardiac defects by inducing protein misfolding, oxidative stress, and autophagy.[16,20,21] However, the precise mechanisms of PolyQ-induced cardiomyopathy remain unknown. Thus, we examined the mechanism by which aggregate accumulation caused abnormal heart function.[7] As detailed below, we found that cardiac expression of mutant PolyQ produced myofibrillar degeneration and ultrastructural defects in a PolyQ length-dependent manner.

Mutant PolyQ and Protein Misfolding

Molecular chaperones reduce the toxicity of protein aggregation by facilitating folding of aggregation-prone mutant HTT.[1] Several chaperones, including Hsp-70, Hsp-40, and Hsp-110, have been shown to modulate HD phenotypes in neurons.[22,23] However, it remains unclear if expression of mutant PolyQ results in the misfolding of contractile proteins in the human heart. Although mutant PolyQ leads to cardiac failure in mice,[16,24] it was previously unknown if PolyQ directly targets accumulation of key contractile proteins, e.g., myosin and actin. As mentioned in our manuscript,[7] a majority of PolyQ mutant hearts contains non-contractile regions, indicative of cell death or mislocalization of contractile proteins. Since the proper folding of such proteins is essential for cardiac function, we sought to determine if the presence of non-contractile regions in the hearts of PolyQ mutant flies is due to mislocalization of contractile proteins.[7] We showed that cardiac expression of mutant PolyQ leads to disruptions in the accumulation of myosin and actin in cardiac myofibrils and that these changes are associated with severe defects in contractility. It is not known if mutant PolyQ leads to myofibrillar abnormalities by interacting with these contractile proteins or by interfering with the unfolded protein response, which is necessary to maintain ER homeostasis and contractile protein turnover (reviewed by Groenendyk et al).[25] We showed that GFP-tagged HTT protein aggregates co-localize with both actin and myosin,[7] suggesting a possible link between aggregate formation and defects in assembly or maintenance of actin and myosin-containing myofibrils. Alternatively, the formation of pathogenic aggregates may also inhibit the unfolded protein response by directly interfering with chaperone activity. In an amyloid model for desmin-related cardiomyopathy, mutant alphaB-crystallin (R120G) expression in cardiomyocytes caused defects in chaperone activity and folding of contractile proteins.[15,21,26] Expression of both mutant alphaB-crystallin and mutant PolyQ resulted in amyloid-positive aggregate formation in cardiomyocytes and myocyte degeneration,[21] suggesting a possible link between protein misfolding and cardiomyocyte death. To determine the role of the unfolded protein

response in PolyQ-induced cardiomyopathy, we overexpressed the myosin-associated chaperone UNC-45 in PolyQ mutants as a novel approach to suppress PolyQ-induced cardiac defects.[7] We showed that overexpression of UNC-45 suppresses PolyQ-induced formation of aggregates, loss of myofibrils, and ameliorated cardiac defects to a great extent, suggesting that protein misfolding plays a role in the loss of myosin due to expression of mutant PolyQ. Although overexpression of UNC-45 in PolyQ mutant hearts restored the integrity of actin-containing myofibrils as well, it remains unclear if the loss of actin-containing myofibrils due to mutant PolyQ is caused by decreased accumulation of myosin, or if PolyQ aggregates interfere with the accumulation of other contractile proteins such as actin. We found that overexpression of UNC-45 alone could not completely suppress cardiac dysfunction. A nearly complete reversal of PolyQ-induced cardiac phenotypes required modulation of the oxidative stress response in addition to UNC-45 overexpression.

Mutant PolyQ and Oxidative Stress

Since the accumulation of misfolded and aggregated proteins is known to induce mitochondrial damage and oxidative stress in several neurodegenerative disorders,[27] we hypothesized that oxidative stress plays a role in PolyQ-mediated cardiac dysfunction. In support of our hypothesis, Mihm et al. showed that cardiac expression of mutant PolyQ was associated with mitochondrial morphological defects and enhanced oxidative stress.[16] Furthermore, impairment of mitochondrial function and decreased expression of electron transport chain proteins have been linked to the presence of misfolded PolyQ aggregates in neurons of HD patients.[28] The majority of reactive oxygen species (ROS) generation is due to leakage of oxidants from the electron transport chain of mitochondria.[29] Since mitochondria occupy greater than 25% of cardiomyocyte volume to provide sufficient energy for contractility,[30]

cardiomyocytes are particularly susceptible to oxidative stress induced by defective mitochondria. Assuming that cardiomyocyte function in Drosophila is heavily dependent on mitochondrial integrity as in mammalian systems,[31] we sought to examine if PolyQ-induced cardiac dysfunction is linked to mitochondrial ultrastructural abnormalities. Cardiac expression of mutant PolyQ-46 led to severe mitochondrial cristae fragmentation, which was observed in electron micrographs of thin sections (**Fig. 1**). In our manuscript,[7] we showed that suppression of PolyQ-induced oxidative stress by overexpressing superoxide dismutase (SOD) was critical to restore functional and structural cardiac abnormalities, including mitochondrial ultrastructural defects. It remains unclear whether myofibrillar degeneration due to aggregation of mutant PolyQ is directly inhibiting mitochondrial organization, and/or if oxidative stress produced by PolyQ leads to mitochondrial dysfunction. Mitochondrial organization is meticulously controlled by the cardiomyocyte and, therefore, depends on the proper function and organization of contractile proteins.[32] Thus, our studies suggest that HD pathogenesis in the heart is linked to enhanced oxidative stress together with mitochondrial abnormalities.

Several studies suggest that certain cardiac amyloidosis-based disorders are associated with oxidative stress and mitochondrial dysfunction. The vulnerability of mitochondria to oxidative stress was shown in a mouse model for oxidative stress-related dilated cardiomyopathy by genetic ablation of the mitochondrial form of superoxide dismutase, SOD_2, which resulted in an abnormal "ballooning" of mitochondria in electron micrographs of dendrites and decreased lifespan.[33] Additionally, cardiac-specific expression of mutant alphaB-crystallin also resulted in the accumulation of amyloid-containing protein aggregates in cardiomyocytes in a cell-autonomous manner, leading to enhanced oxidative stress.[15,32,34] Decreasing SOD production in alphaB-crystallin mutants by pharmacologically inhibiting the activity of the superoxide-generating xanthine oxidase restored mitochondrial function and membrane potential, suggesting a link

between mitochondrial dysfunction and oxidative stress due to cardiac amyloidosis.[34] Thus, enhanced oxidative stress and mitochondrial dysfunction leading to heart failure may depict a common mechanism among cardiac amyloidosis-based disorders.

Multiple Cellular Pathways are Involved in HD Cardiac Pathogenesis

In our manuscript,[7] we manipulated both the oxidative stress and protein misfolding pathways as a novel approach to suppressing PolyQ-induced cardiac phenotypes. We found that overexpression of UNC-45 or SOD alone did not completely suppress mutant PolyQ-induced cardiac phenotypes, suggesting that multiple cellular pathways are involved in HD cardiac pathogenesis. Our studies demonstrated that overexpression of SOD in conjunction with the myosin-associated UNC-45 chaperone led to better suppression of PolyQ-induced cardiac dysfunction *in vivo* than either treatment alone. Overexpression of both was required to improve fractional shortening and decrease arrhythmias in PolyQ-72 mutants nearly to the extent of driver line controls. Additionally, this combined treatment reduced aggregation of mutant GFP[+] PolyQ punctae in PolyQ-72 mutant hearts more than either treatment alone. We did not show whether ultrastructural phenotypes in PolyQ mutant hearts were suppressed with overexpression of UNC-45 and SOD in our manuscript.[7] Here, we compared thin sections of hearts from 4 week-old flies in mutant and suppressor lines, using transmission electron microscopy to determine if combined overexpression suppresses ultrastructural defects caused by expression of mutant PolyQ (**Fig. 1**). Expression of mutant PolyQ-46 resulted in severe disruptions in mitochondrial cristae structure and myofibrillar degeneration, which were suppressed with UNC-45 and SOD overexpression. Hearts expressing both suppressors contained normally shaped mitochondria with dense cristae as well as reduced myofibrillar degeneration (arrow). In support of our findings, PolyQ
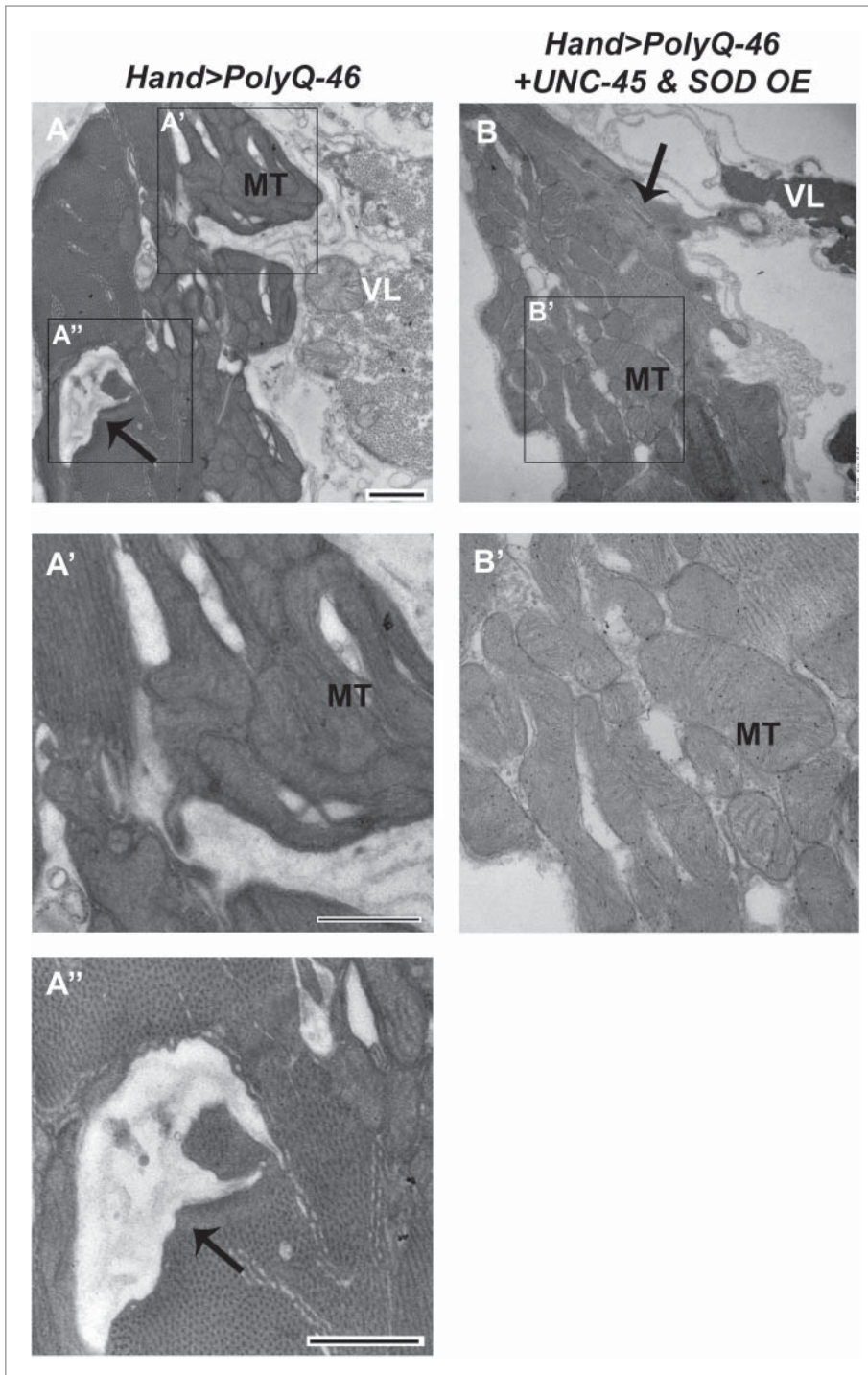
Figure 1. Mitochondrial ultrastructural defects in mutant PolyQ-expressing hearts are suppressed with UNC-45 and SOD overexpression. (**A, A′, A″**) Cardiac expression of mutant PolyQ-46 resulted in degenerated areas within the cardiomyocyte (arrow), along with fragmentation of mitochondria in cardiomyocytes. MT indicates a fragmented mitochondrion. (**B, B′**) Combined overexpression of SOD and UNC-45 in PolyQ-46-expressing hearts improved mitochondrial ultrastructure. MT indicates a normally shaped mitochondrion with densely packed cristae. Degeneration (arrow) was reduced to some extent in SOD+UNC-45 overexpressing hearts. VL refers to non-cardiac ventral-longitudinal fibers. Scale bar is 500 nm.

aggregation in mouse neurons was enhanced *in vitro* with oxidative stress and suppressed with overexpression of SOD together with chaperones Hsp-70/Hsp-40,[35] suggesting that targeting both cellular pathways is efficacious in HD pathogenesis in multiple tissue types. Our results suggest that both the oxidative stress and protein unfolding pathways play a role in PolyQ-induced cardiac pathogenesis, and targeting both pathways is a relevant approach for suppressing PolyQ-induced cardiomyopathy.[7] It is unknown whether combined overexpression of UNC-45 and SOD rescues cardiac defects in other cardiac amyloidosis disorders. If the cellular pathways governing other cardiac amyloidosis disorders are similar to those involved in PolyQ-induced cardiomyopathy, our model can provide valuable insight into potential disease mechanisms in other models and relevant therapeutic approaches. However, some aggregation-based cardiac diseases may not involve mitochondrial dysfunction. Thus, further studies are necessary to determine the broad implications of manipulating both pathways to treat other cardiac amyloidosis disorders.

Future Directions

Further investigation will explore the exact molecular players associated with the accumulation of PolyQ-induced cardiomyopathy (**Fig. 2**). Based on our findings, we propose a model in which mutant PolyQ is associated with aggregate formation in the heart, possibly causing mitochondrial abnormalities associated with enhanced ROS. Although other studies (reviewed by Taverne et al.)[36] have also suggested a role for ROS in inducing cardiac dysfunction, it is unknown if accumulation of PolyQ aggregates generates ROS by interfering with mitochondrial function directly, and/or if mitochondrial damage is a result of ROS generation due to PolyQ aggregation. Although not tested in our model, we speculate that some mutant PolyQ-induced cardiac defects are due to mitochondrial dysfunction independent of PolyQ aggregation. Further studies are necessary to determine if cardiac defects occur in the absence of aggregate formation. Additionally, it is unknown whether enhanced oxidative

stress and mitochondrial dysfunction are causative of cardiac dysfunction or markers of disease. The exact mechanisms regulating mitochondrial damage in association with ROS will be deciphered in future studies. Although ROS generation is known to promote PolyQ aggregation in HD 150Q cultured cells, causing proteosomal defects and mutant HTT aggregation,[35] it is unknown if decreased proteosomal function plays a role in exacerbating PolyQ aggregation in cardiomyocytes.

The molecular mechanisms whereby UNC-45 suppresses cardiac dysfunction and myofibrillar/mitochondrial abnormalities due to mutant PolyQ remain unclear. However, protein folding to enhance myofibrillar integrity. It is well-established that UNC-45 promotes the assembly and refinement of the contractile apparatus by directly binding and folding myosin to enhance thick filament assembly as well as cardiac contractility.[37-40] However, it remains unknown if UNC-45 directly prevents the formation or mediates the clearance of PolyQ aggregates to promote refolding of mutant HTT protein. Although UNC-45 is known to bind other chaperones including Hsp-70 and Hsp-90 in vitro,[41,42] it is unknown if these proteins act as co-chaperones to promote refolding of mutant HTT protein. Further studies will determine if UNC-45 directly interacts with contractile proteins to suppress myofibril degeneration or assembly defects due to mutant PolyQ, and/or if UNC-45 can inhibit formation or mediate clearance of PolyQ aggregates through interactions with other chaperones.

We are interested in additional cellular processes involved in PolyQ-induced cardiomyopathy. In PolyQ-72 mutant hearts, Lysotracker[+]-positive punctae co-localized with GFP[+] aggregates, suggesting a possible role of autophagy in the clearance of PolyQ aggregates. In support of our model, a non-HTT mouse cardiac model for PolyQ repeat expansion disorders showed increased autophagy associated with aggregation of mutant PolyQ and heart failure.[43] Further studies are necessary to confirm the enhanced formation of autophagosomes in mutant HTT hearts and their potential roles in the clearance of protein aggregates, defective
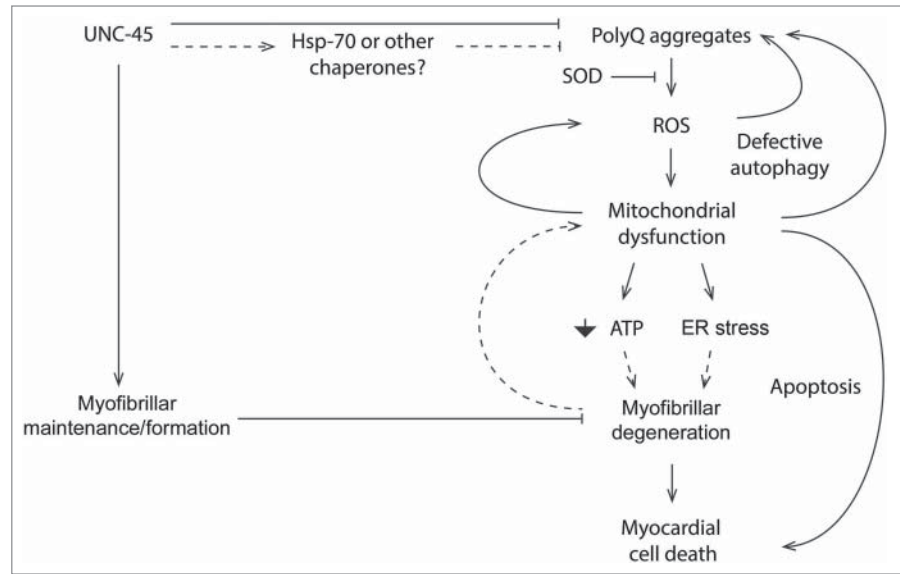

Figure 2. Mechanisms of PolyQ-induced cardiac amyloidosis. Our findings support a model in which both the oxidative stress and protein unfolding pathways underlie cardiac pathogenesis arising from mutant PolyQ. Future approaches will aim to characterize the exact molecular players associated with the production of PolyQ-induced cardiomyopathy.

mitochondria, or both. In mice, cardiac expression of mutant PolyQ led to defects in autophagy-mediated clearance of defective mitochondria.[21] In our model, mitochondrial ultrastructural defects were observed in PolyQ mutant hearts, which were suppressed with SOD overexpression, suggesting that mitochondrial dysfunction and ROS generation play a role in myofibrillar degeneration.[7] Thus, modulating the autophagy-mediated clearance of defective mitochondria may become a useful therapeutic strategy for future approaches to combat PolyQ-induced cardiomyopathy.

Since mitochondria occupy a large portion of cardiomyocyte volume to provide sufficient energy for contractility,[30,44] myofibrillar degeneration and disorganization in PolyQ mutant hearts may occur due to defective ATP generation by dysfunctional mitochondria or enhanced ER stress. Alternatively, mitochondrial dysfunction may trigger activation of apoptotic pathways, causing cell death in PolyQ mutant hearts. The presence of non-contractile regions in mutant PolyQ hearts suggests that myocardial cell death plays a role in cardiac pathogenesis, and further studies will determine if cell death occurs through defects in energy generation or by activation of apoptotic pathways.

In conclusion, we demonstrated that modulating both the protein unfolding and oxidative stress pathways suppress PolyQ-induced cardiac defects in a Drosophila model. Additional cellular pathways that underlie cardiac failure in HD patients have not been explored and will provide a basis for developing further therapeutic interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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