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Studying Protein Aggregation in PolyQ disease with Proof-of-Concept Zebrafish Assays

Beclin-1, TFEB and MDM2 Axis

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Introduction:

PolyQ diseases are abnormal expansion of CAG repeats in the genome leading to an inheritable form of neurodegenerative disease caused by toxic gain of function. A number of avenues of rescue of PolyQ diseases are currently being investigated such as aggregate reduction, promotion of proteasome pathways, cellular transplants, protein degraders, reactive oxygen species inhibition among many others. However therapeutic progress is still very much at infancy. Much of the hurdles in PolyQ diseases are due to limitations within model systems that do not provide precise feedback on which molecular axis is regulated.

> “In zebrafish models of PolyQ disease, mutants of molecular targets provide precise feedback on pathway modulation.”

This is due to fact that bi-allelic mutant lines of pathways can be quickly generated and screened along with study groups to provide feedback if the mutant line is also rescued or not, thereby validating the mechanism of therapeutic agent under study. This feedback driven model system can be a surreal indicator of therapeutic agent activity and help navigate the discovery landscape for PolyQ diseases.

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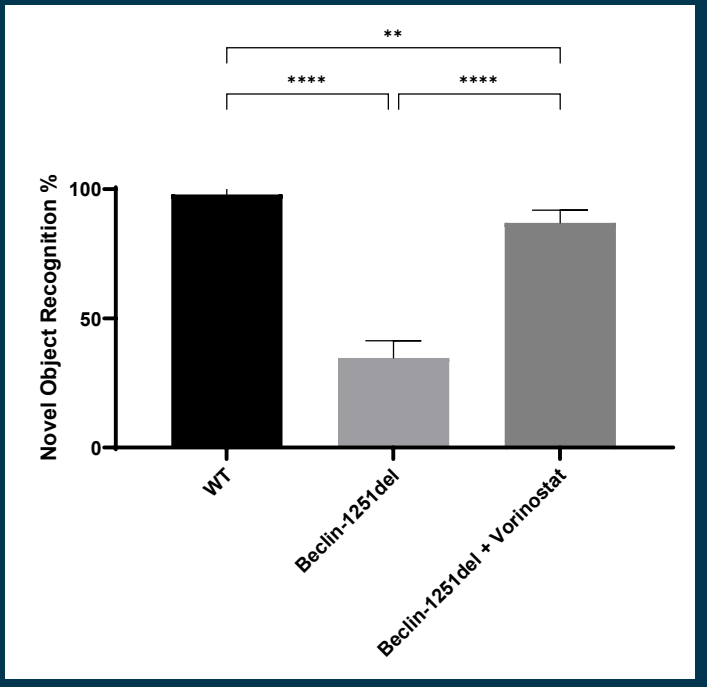
Beclin–1 Axis

Beclin-1 is a key protein necessary for successful autophagy. Beclin-1 plays a critical role in autophagosome in the nucleation step where it forms a core complex. This is subsequently followed by elongation and maturation steps, leading to a successful protein degradation. In polyQ diseases beclin-1 interacts with multiple proteins through polyQ sequences; pathological proteins such as mutant huntingtin, ataxin-3, atrophin-1 and the androgen receptor interact with beclin-1 to undergo autophagy.

Overexpression of beclin-1 has demonstrated rescue effect in rodent models in early Huntington pathology and several other autophagosome disorder driven pathologies. In zebrafish models of beclin-1 partial mutants that show reduced functional activity, animals developed neurodegenerative phenotype of reduced memory and learning. These phenotypes were significantly rescued in early course of disease with vorinostat, a histone deacetylation inhibitor.



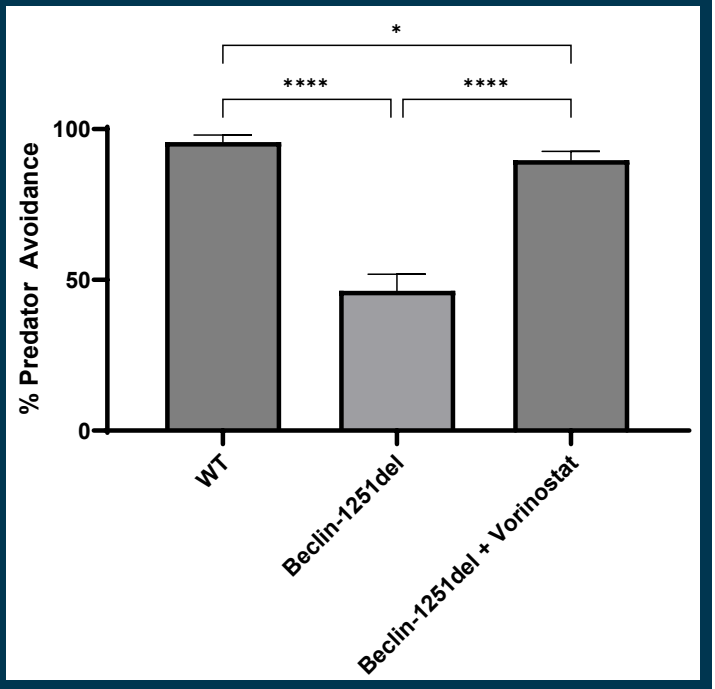


Image 1: Graph showing improvements in Memory – Novel Object Recognition Test and Learning – Predator Avoidance Test, when beclin-1 deficient animals are dosed with histone deacetylation inhibitor, vorinostat.

TFEB – Axis

Mutation of the ataxin-3 gene results in spinocerebellar ataxia (SCA). Ataxin-3 functions to regulate genome stability, transcription, cellular stress and is a deubiquitinating enzyme. Mutant ataxin-3 forms with CAG repeats, results in the hereditary neurodegenerative disease, SCA. Pathogenic forms of ataxin-3 accumulate in the vulnerable regions of the brain leading to selective neurodegeneration in different regions of the brain. Toxicity of ataxin-3 has been rescued by antisense oligos *in vitro* and in mice models. In *C.elegans*, inhibitors of oxidative stress such as salubrinal have demonstrated rescue of ataxin-3 toxicity.

In zebrafish, ataxin-3 mutant treated with digoxin a positive modulator of TFEB dependent Autophagy-Lysosomal Pathway acutely rescued limb ataxia and memory phenotypes

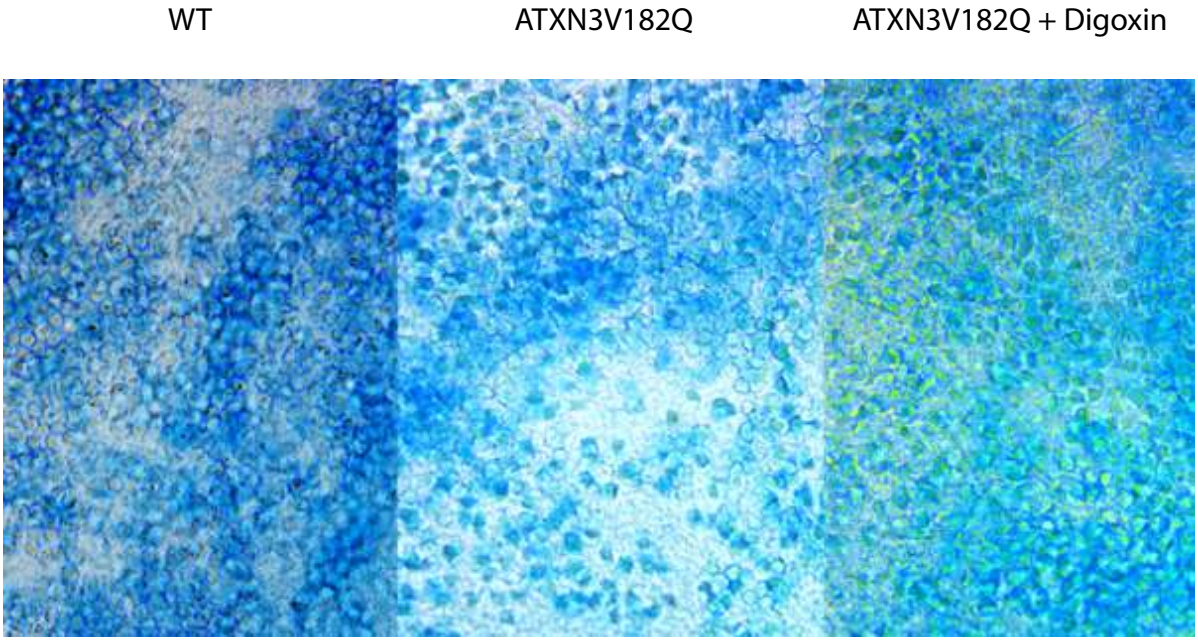


Image 2: **Sections of brain from spinocerebellar ataxia model showing accumulation of aggregates in neuronal cell body of SCA mutants. Method: Viable brain slice staining with methylene blue, captured with bright field microscopy at 40X.**

Watch video of viable brain slices at
www.youtube.com/watch?v=HwLv-Dtr3Ik

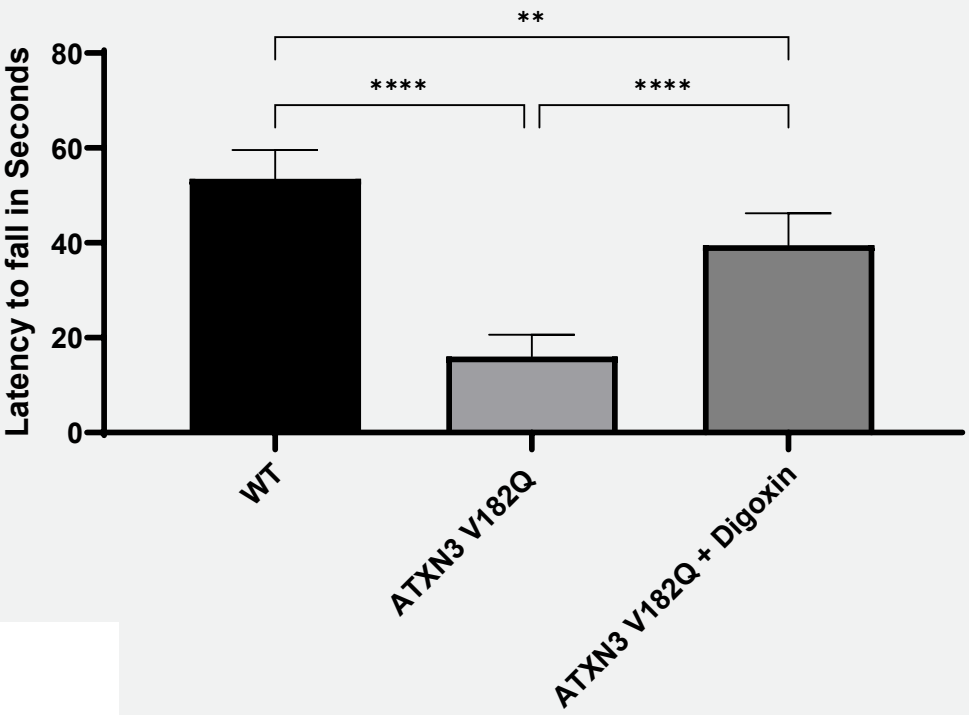
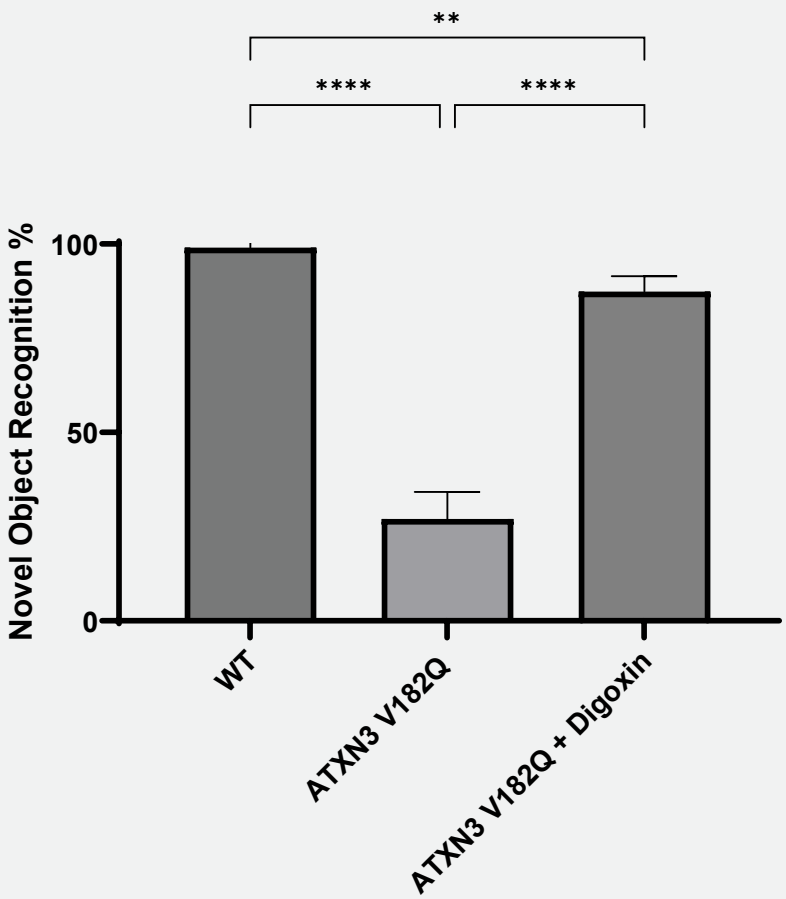


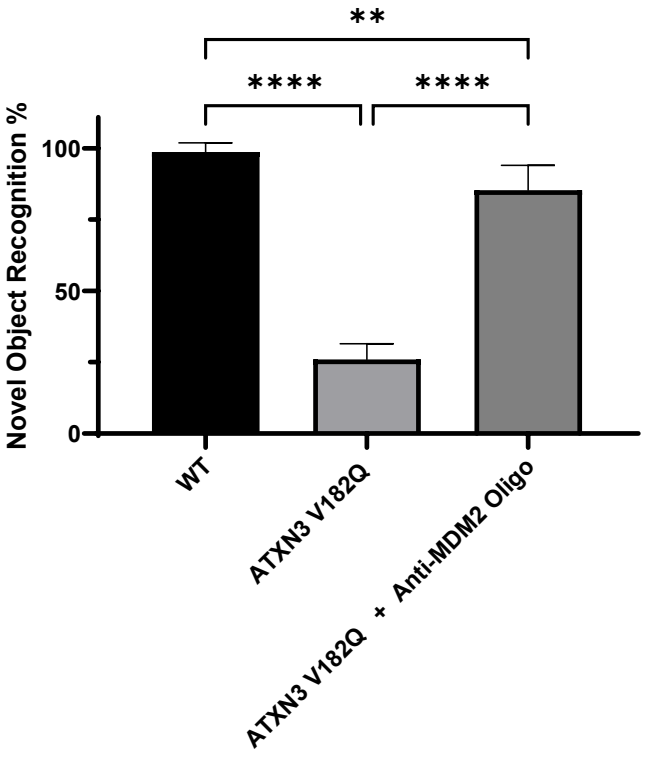
Image 3: Graph showing improvements in Limb ataxia – Swim Tunnel Test, Memory – Novel Object Recognition Test, in SCA mutants when dosed with digoxin a positive modulator of TFEB dependent Autophagy-Lysosomal Pathway



MDM2 Axis

MDM2 is a negative regulatory of p53 and is implicated in several diseases including inflammation and neuronal death. MDM2 inhibition has been demonstrated in neurogenic rescue and improved novel object recognition in rodent model of fragile X syndrome. MDM2 inhibited rescue of autophagy has been demonstrated in several *in vitro* studies. Other studies summate MDM2 inhibition to rescue synaptic loss driven by amyloid beta (Aβ) and retain brain plasticity.

In zebrafish SCA model, inhibition of MDM2 rescued SCA pathology of memory and learning likely by induction of p53 dependent autophagy.



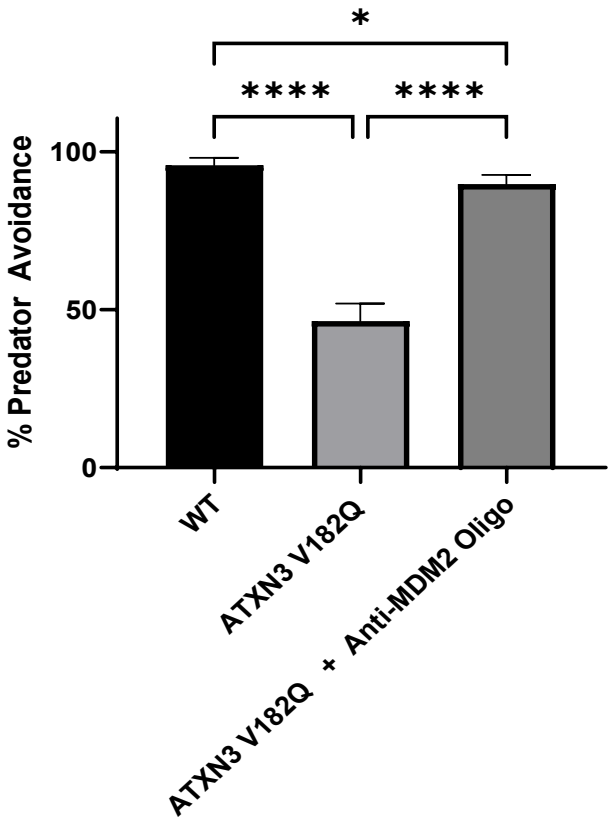


Image 4 : Graph showing improvements in Memory – Novel Object Recognition Test and Learning – Predator Avoidance Test, in SCA mutants on inhibition of MDM2.

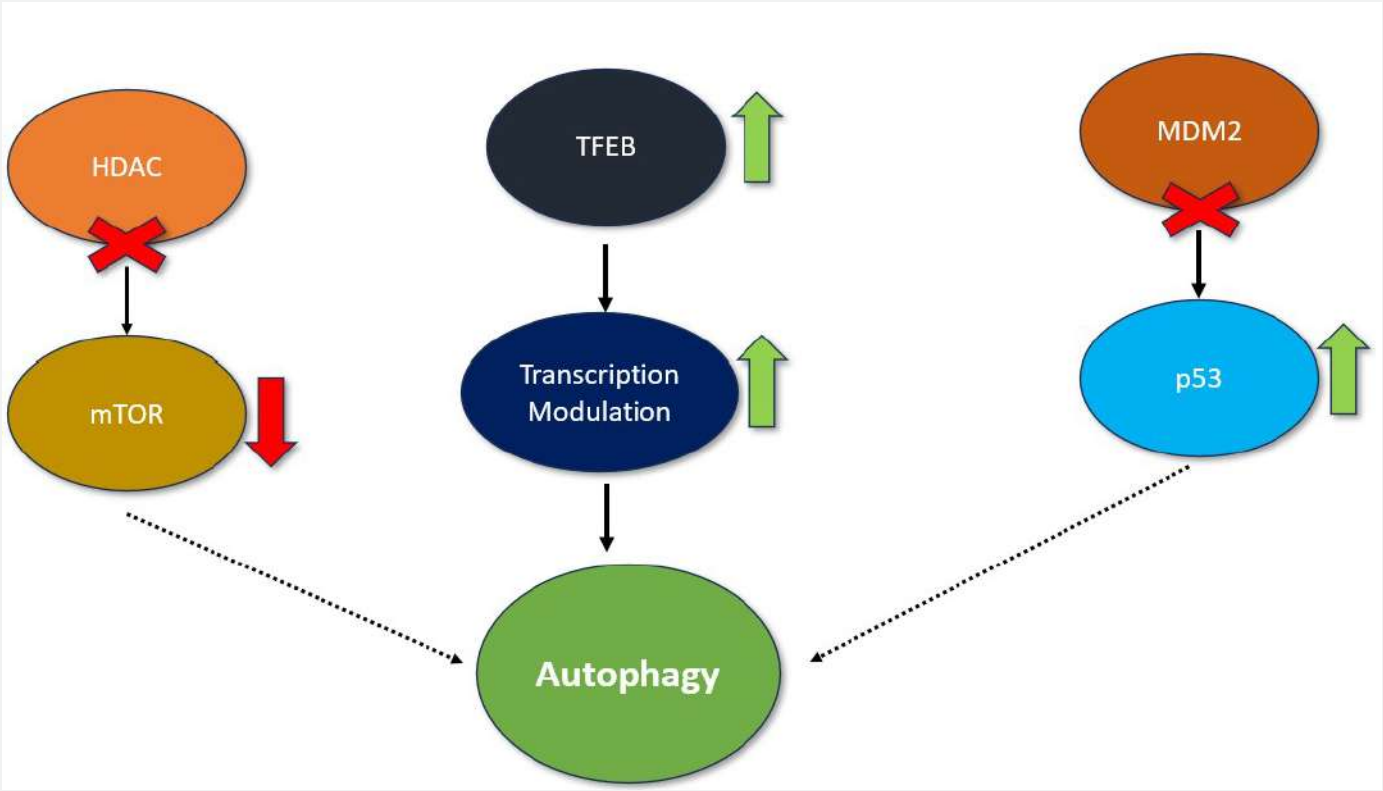


Image 5: Schema showing pathways in PolyQ discovery deciphered by zebrafish mutants.

Assays – Novel Object Recognition for Memory

The study fishes were transferred to the experiment tank of 30X19 (LXB) cm divided into 2 sections with a marker. The study subject was introduced to the tank with a static object (blue coloured ball), the primary study object at one zone of the tank. Individuals were left to acclimatize for 30 min and were released back to the housing tank and were maintained for 30 min post which the larvae were reintroduced to the experimental tank. During this re-exposure period a novel object- blue colour triangular block (1X1 cm) was introduced in to the other zone of the tank and was kept static.

Study fishes were observed for the time spent in the either section of the tank. Observations were recorded and were exported to microsoft excel. An aerial view of the tank was captured using Apple iPad. Zebrafish is good at exploring new areas post acclimatization with the familiar zone. In the given 15 min observation duration, an extended time spent in the familiar object zone ie the primary object- blue colored ball, rejecting the novel object indicates good memory retention. The study used only 2 dimensional objects as zebrafish possess recognition memory for simple 2- and 3-dimensional geometrical shapes.

Assay - Predator avoidance assay for Learning

This is a fear-aggravated test to evaluate spatial memory retention in response to threat. In this experimental set-up, an adult male zebrafish (cannibalistic) was used as a predator to create a threatening stimulus. The complete experimental set was designed to be transparent to create an optical illusion of no escape channel. The tank was partially divided to two sections with the predator tank on the left. To study predator avoidance behavior, the 21 dpf larvae were screened in two phases: Habituation and Test

Test-predator escape and avoidance behaviour

The larvae were employed for assessment within 2 hours of habituation. During the test, the larvae was released at the start point of the experimental tank and was observed for predator avoidance behavior. Larvae that exhibited spatial memory retention avoided the left section of the experimental tank, which housed the predator. Larvae that continued to explore the left section of the tank were considered to show reduced spatial memory.

Thread Mill Assay for Ataxia Measure

In this study, swim tunnel assay was performed to investigate the motor coordination of the larvae by determining the latency of the larvae against the flow velocity of the water in the swim chamber. To study the motor coordination larvae were introduced to the swim chamber (4 cm length and 4 mm diameter) and was allowed to acclimatize for 5 minutes before screening. Following acclimatization, water inflow began through a multi-aperture channel and outflows through a grid. The rate of water inflow and outflow in the chamber was adjusted and a velocity of 3 BLS-1 (where 1 body length = 6 mm) was set. The larvae were subjected to swim against the inflowing water current in the chamber.

The time taken by the larvae to fatigue and fall in the outflow rear of the chamber denotes the motor coordination latency to fall by the larvae. During the assessment resting (by clinging to surface) and wobbling time of the zebrafish larvae in the chamber was excluded from the total latency.

Conclusion: Most of the successful *in vitro* studies suffer from translation barriers; zebrafish mutants can ensure translation by not only linking dose to effect results, but linking specific molecular event to effect which is a far more precise method of translation to clinical studies.