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Supplemental Information

Unravelling Endogenous MicroRNA System Dysfunction as a New Pathophysiological Mechanism in Machado-Joseph Disease

Vitor Carmona, Janete Cunha-Santos, Isabel Onofre, Ana Teresa Simões, Udaya Vijayakumar, Beverly L. Davidson, and Luís Pereira de Almeida

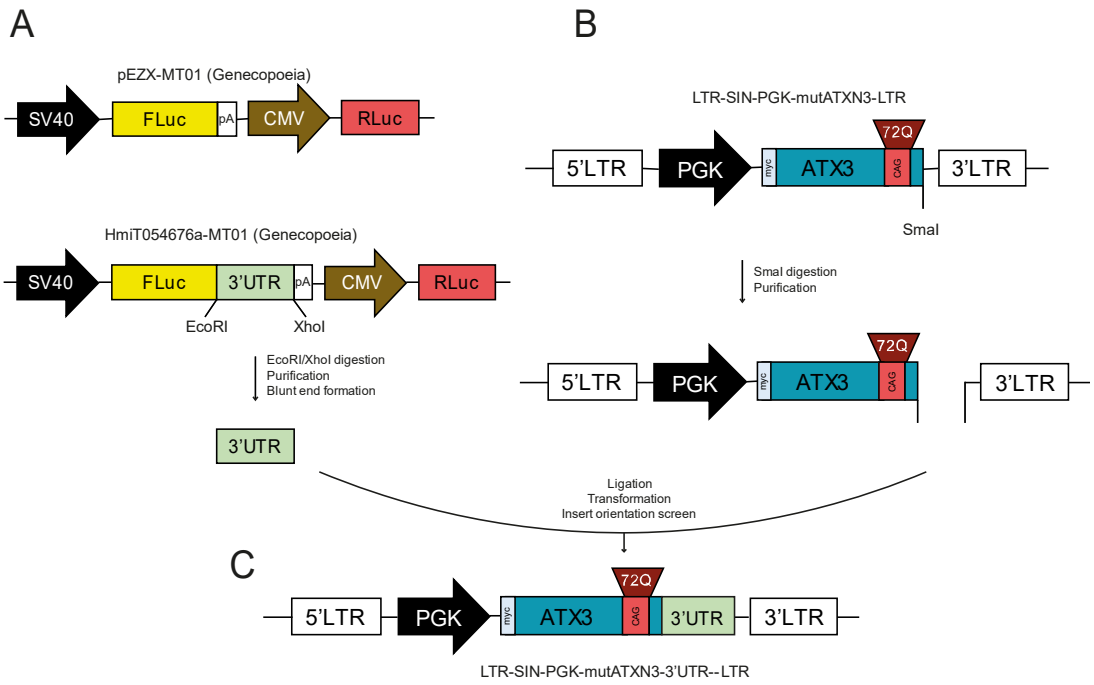

Figure S1. Schematic representation of the procedure used to clone human ATXN3 3'UTR in a lentiviral vector. (A) Plasmid constructions encoding for firefly luciferase bound or not to human ATXN3 3'UTR were purchased from Genecopoeia. ATXN3 3'UTR was extracted from HmiT054676a-MT01 after EcoRI and XhoI digestion. The insert was later purified, and turned blunt using Klenow polymerase. (B) A lentiviral vector encoding for mutATXN3 (LTR-SIN-PGK-mutATXN3-LTR) was used as backbone for the introduction of ATXN3 3'UTR after being digested with SmaI. (C) The 3'UTR fragment was ligated downstream of ATXN3 CDS in order to generate LTR-SIN-PGK-mutATXN3-3'UTR-LTR.

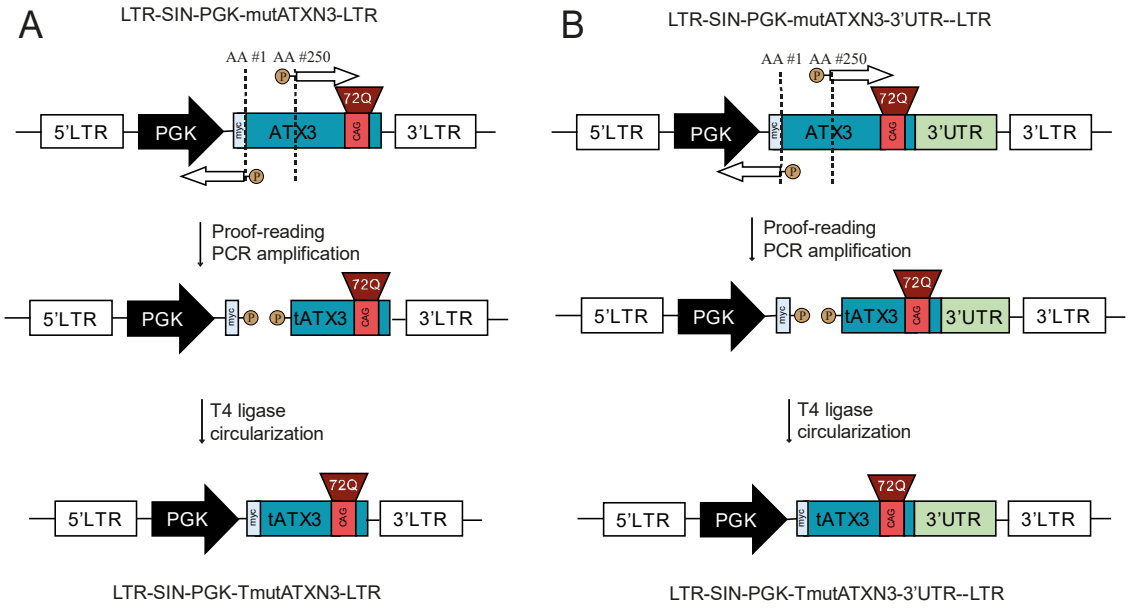

Figure S2. Schematic representation of the procedure used to generate truncated isoforms of mutATXN3. (A) LTR-SIN-PGK-mutATXN3-LTR was used as PCR template for inverted PCR mutagenesis. Two phosphorylated oligonucleotides were designed in order to amplify the parental vector full sequence with the exception of the sequence correspondent to the first 250 amino acids of ATXN3. Linear PCR products were circularized with T4 DNA ligase in order to generate LTR-SIN-PGK-TmutATXN3-LTR. (B) LTR-SIN-PGK-mutATXN3-3'UTR-LTR was used as PCR template for inverted PCR mutagenesis. Two phosphorylated oligonucleotides were designed in order to amplify the parental vector full sequence with the exception of the sequence correspondent to the first 250 amino acids of ATXN3. Linear PCR products were circularized with T4 DNA ligase in order to generate LTR-SIN-PGK-TmutATXN3-3'UTR-LTR.

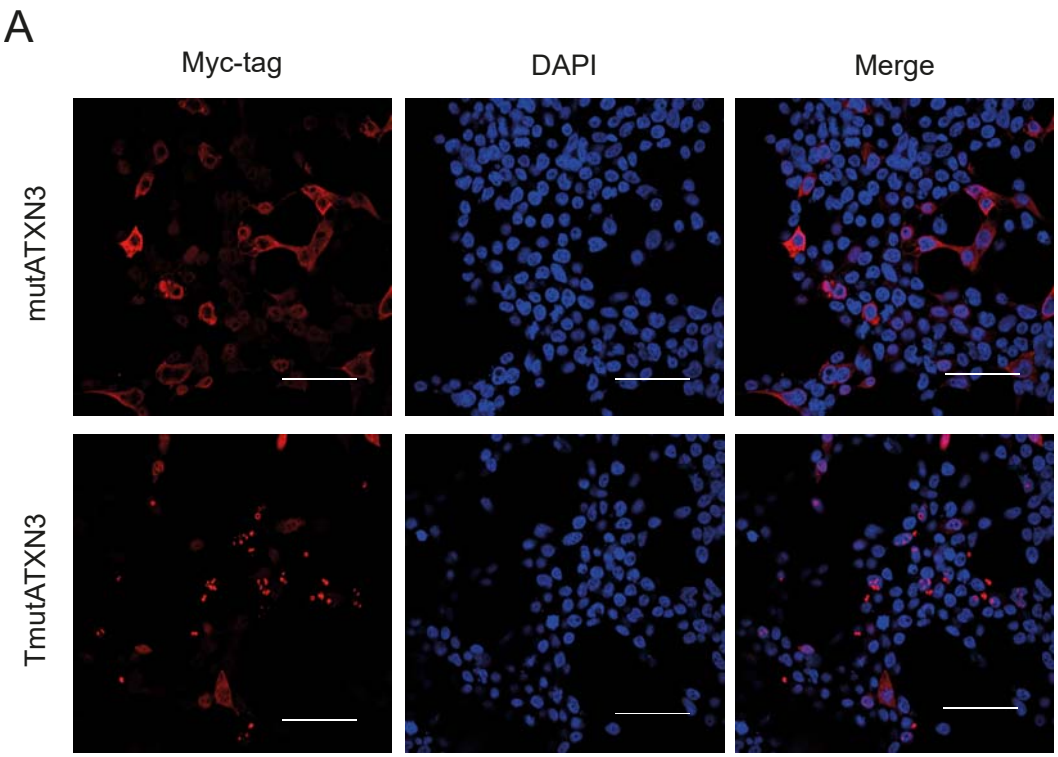

Figure S3. Generation of constructs encoding for a truncated form of mutATXN3 enhance aggregation *in vitro*. (A) Truncated versions of mutATXN3 (TmutATXN3) were constructed in order to promote aggregation of mutATXN3 in vitro. Upon transfection of HEK29T cells, full-length mutATXN3 remains mostly cytoplasmatic with a diffuse staining while TmutATXN3 results in extensive formation of characteristic intranuclear mutATXN3 inclusions. Scale bars: 50µm.

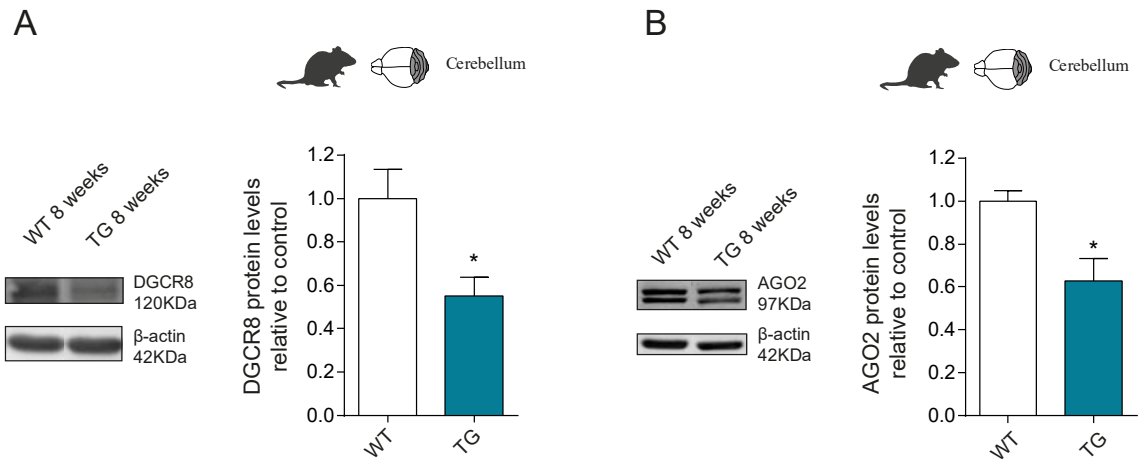

Figure S4. DGCR8 and AGO2 protein levels are decreased in MJD transgenic mice. (A) DGCR8 protein levels were found to be significantly downregulated in MJD transgenic mice when compared to littermate control animals (n=5/5). (B) AGO2 protein levels were found to be significantly downregulated in MJD transgenic mice when compared to littermate control animals (n=4/5). All samples were normalized with actin. Statistical significance was evaluated with unpaired Student's t-test (*P<0.05). Data are expressed as mean ± SEM.

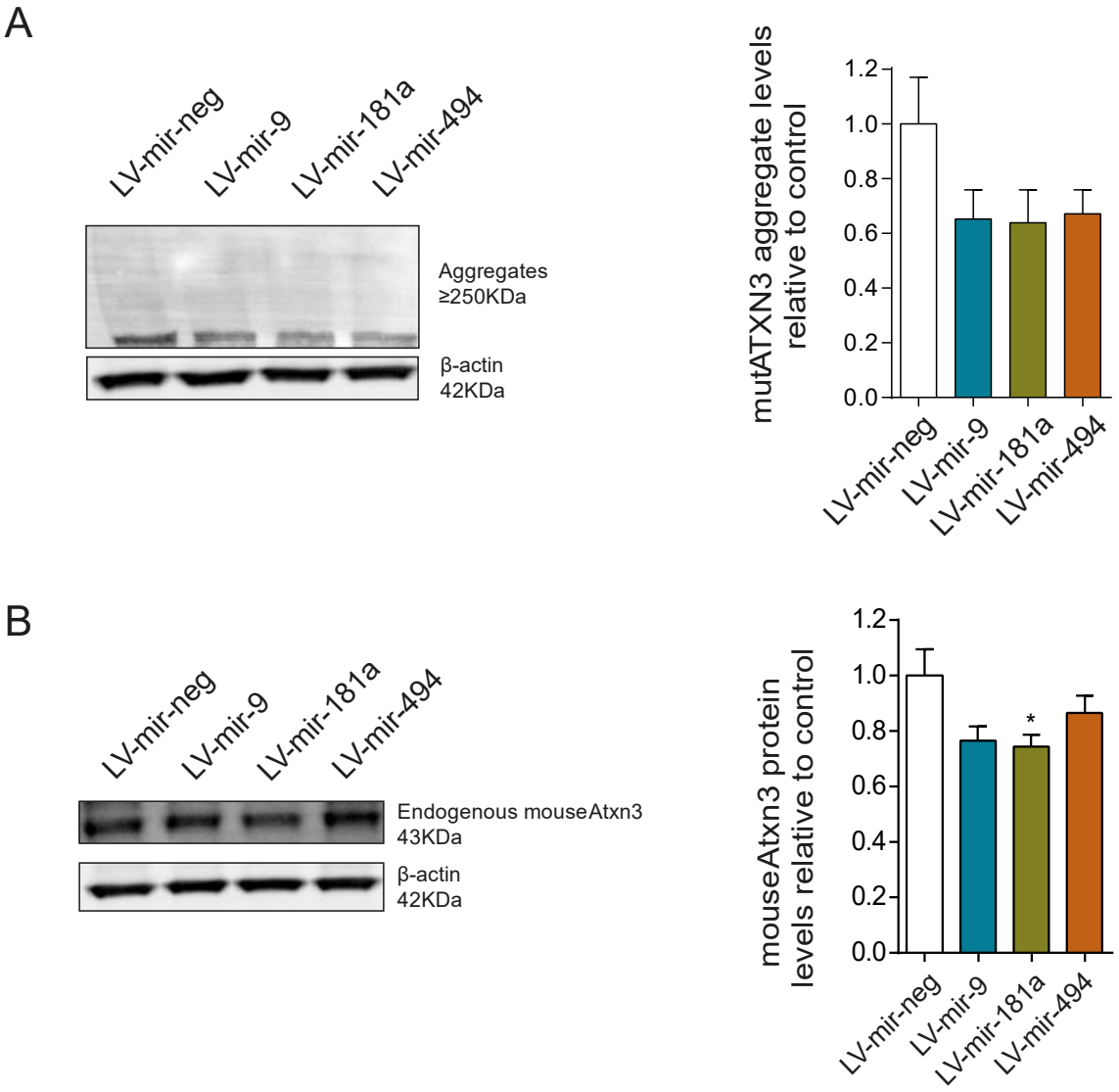

Figure S5. Western blot analysis of human mutATXN3 and endogenous mouse Atxn3 *in vivo.* (A) Stereotaxic injection of lentivirus encoding for mutATXN3 together with either mir-9, mir-181a or mir-494 led to a tendency for a reduction in the levels of aggregated mutATXN3 when compared to animals injected with mir-neg (n=4-5). (B) The levels of endogenous mouse Atxn3 were found to be significantly downregulated in mice injected with lentiviral particles encoding for mir-181a. This is in accordance with the existence of a miRNA binding site for mir-181a in the 3'UTR of mouse Atxn3 (n=4-5). All samples were normalized with actin. Statistical significance was evaluated with unpaired Student's t-test (*P<0.05). Data are expressed as mean ± SEM.

Table S1. Predicted miRNA binding sites for mir-9, mir-181a and mir-494 in human ATXN3 3'UTR.

MicroRNA	Binding site	3'UTR position
hsa-mir-9-5p	CDS ··· UUAGCCAAAGAG ··· Poly(A) 3' ··· UCUAUUGGUUUCU - 5' miRNA	**190-196**
hsa-mir-181a-5p	CDS ···CUUUAUGAAUGUC··· Poly(A) 3' ··· UCGCAACUUACAA 5' miRNA	**770-776**
hsa-mir-181a-5p	CDS ···CACCAUGAAUGUA··· Poly(A) 3' ··· UCGCAACUUACAA 5' miRNA	**929-936**
hsa-mir-181a-5p	CDS ···UCAUGGAAUGUAG··· Poly(A) 3' ··· UCGCAACUUACAA 5' miRNA	**1033-1039**
hsa-mir-181a-5p	CDS ···UCUUUUGAAUGUU··· Poly(A) 3' ··· UCGCAACUUACAA 5' miRNA	**1381-1387**
hsa-mir-181a-5p	CDS ···GCGUUUGAAUGUG··· Poly(A) 3' ··· UCGCAACUUACAA 5' miRNA	**2393-2399**
hsa-mir-494-3p	CDS ··· UAAAAAUGUUUCA··· Poly(A) 3' ··· GCACAUACAAAGU- 5' miRNA	**236-242**
hsa-mir-494-3p	CDS ···UCUAAAUGUUUCU··· Poly(A) 3' ··· GCACAUACAAAGU- 5' miRNA	**1696-1703**

Table S2. List of oligonucleotide sequences used for PCR based cloning of constructs encoding for pre-miRNA and negative control.

microRNA	**Primer Sequence** (BglII/HindIII)		**Amplicon size**
hsa-mir-9	Forward	GTGAT AGATCT GAAATGTCGCCCGAACCAGT	399
	Reverse	GTGAT AAGCTT TTGTTGTTTTGTCTCGGACTTCA	
hsa-mir-181a	Forward	TGATCAGATCTACTGCACAGTCTATCCCACAGTT	400
	Reverse	TGATCAAGCTTAGGAACAGTGAGCAGTAGGAATAA	
hsa-mir-494	Forward	GTGAT AGATCT GACACGCAAATAGAAGCCATCTG	350
	Reverse	GTGAT AAGCTT GCCACACCCCCACGAC	
mir-neg	Forward	GTGAT AGATCT CTGGAGGCTTGCTGAAGGCT	132
	Reverse	GTGAT AAGCTT GGCCATTTGTTCCATGTGAG	

Table S3. List of oligonucleotide sequences used for oligo based cloning of constructs encoding for shRNAs targeting human Dicer and Drosha.

shRNA	Oligonucleotide sequence	
hDicer	Top	GATCCCCGCTCGAAATCTTACGCAAATATTCAAGAGATATTTGCGTAAGATTTCGAGCTTTTTA
	Bottom	AGCTTAAAAAGCTCGAAATCTTACGCAAATATCTCTTGAATATTTGCGTAAGATTTCGAGCGGG
hDrosha	Top	GATCCCCCCAGCGTCCATTTGTACTATTTTCAAGAGAAATAGTACAAATGGACGCTGGTTTTTA
	Bottom	AGCTTAAAAACCAGCGTCCATTTGTACTATTTCTCTTGAAAATAGTACAAATGGACGCTGGGGG

Table S4. List of custom designed oligonucleotides sequences used in quantitative PCR analysis

Gene	Primer Sequence		Amplicon size
hDICER	Forward	TTAACCTTTTGGTGTTTGATGAGTGT	98
	Reverse	GCGAGGACATGATGGACAATT	
hDROSHA	Forward	AACCCTGGGACGAAACCAAG	118
	Reverse	TCAACTGTGCAGGGCGTATC	
WPRE	Forward	CCGTTGTCAGGCAACGTG	86
	Reverse	AGCTGACAGGTGGTGGCAAT	
Albumin	Forward	GCTGTCATCTCTTGTGGGCTGT	139
	Reverse	ACTCATGGGAGCTGCTGGTTC	
hATXN3	Forward	ACAGCATAGGGTCCACTTTGG	103
	Reverse	CAACCGACGCATTGTTCCAC	
18S	Forward	CTCAACACGGGAAACCTCAC	110
	Reverse	CGCTCCACCAACTAAGAACG	
mDGCR8	Forward	GCGAAGAATAAAGCTGCCCG	112
	Reverse	TGTGGTTAAAATACTCCAGTTCTTC	
mFMR1	Forward	AAAGCGAGCCCACATGTTGAT	109
	Reverse	CTGCCTTGAACTCTCCAGTTG	
mDDX6	Forward	GAGTCGAGCTACTCGCCAAG	88
	Reverse	CGATTTCGATGTTCCTGCCTC	
mAGO2	Forward	ACGCTCTGTGTCAATACCCG	100
	Reverse	TCCTTCAGCGCTGTCATGTT	
mTARBP2	Forward	GGACACTCCTGTCGTTGCTG	73
	Reverse	CATGGGAGGGCTCCTGGTTA	