Molecular interaction of circMbl and MBL *in-vivo*

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in

Molecular and Cell Biology

by

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Acknowledgements

I thank the Almighty God I believe in, for his steadfast love and comfort that he has shown through the people around me and making the things possible for me that are deemed to be impossible.

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Abstract

Molecular interaction of circMbl and MBL *in-vivo*

A thesis presented to the Graduate Program in Molecular and Cell Biology

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CircRNAs are evolutionarily conserved class of RNAs produced by a process named back-splicing to form covalently closed loop structures lacking 3' & 5' ends. Recent reports emphasize the role of a subset of circRNAs in gene regulation likely by binding and sponging miRNAs, and their ability to get translated in neural tissues. Moreover, there have been increased reports on the biogenesis of circRNAs. The Kadener lab showed that circRNA biogenesis competes with pre-mRNA splicing. This is particularly evident in the *muscleblind* (*mbl*) locus. For example, in *Drosophila* S2 cell lines MBL itself facilitates the circularization of its own 2nd exon to produce circMbl. MBL binds to several binding sites on the flanking introns and 2nd exon of *mbl* pre-mRNA. Out of many isoforms produced from *mbl* locus, MBL-A and MBL-C, specifically facilitates the biogenesis of circMbl. This thesis demonstrates the differential regulation of circMbl biogenesis *in vivo* by the overexpression of different *mbl* isoforms ubiquitously and locally in fly neurons using Gal4-UAS system. Additionally, there have been reports on the role of MBL in muscular dystrophy due to RNA toxicity caused by the expression of CTG repeats. It has been reported that MBL is sequestered by the CTG repeats and form nuclear foci. This thesis also demonstrates how circMbl expression is regulated in Myotonic Dystrophy models in *Drosophila* alongside characterizing the subcellular localization of mbl-A, mbl-B and mbl-C isoforms in S2 cells and in MD1 models.

Table of Contents

List of Figures

Introduction:

Muscleblind-like (MBNL) protein family consists of RNA binding proteins that are implicated to involve in alternative splicing. In humans, this family consists MBNL1, 2, 3 which have different pre-mRNA targets for alternative splicing activity including cardiac Troponin (cTNT) and insulin receptor (IR) (Thai H Ho, Nicolas Charlet-B, Michael G Poulos, Gopal Singh, Maurice S Swanson, 2004). It has been demonstrated that the alternative splicing of these two pre-mRNAs are misregulated in Myotonic Dystrophy type1. In MD1 patients, the mutant DMPK gene that has C(C)TG repeats cause RNA toxicity by sequestering MBNL protein to form nuclear foci and thus affecting their splicing activity(Kreipke, Kwon, Shcherbata, & Ruohola-Baker, 2017). In *Drosophila*, all these functions are carried out by a single *muscleblind* gene that uniquely encodes several isoforms. On FlyBase, it has been reported that 13 transcripts

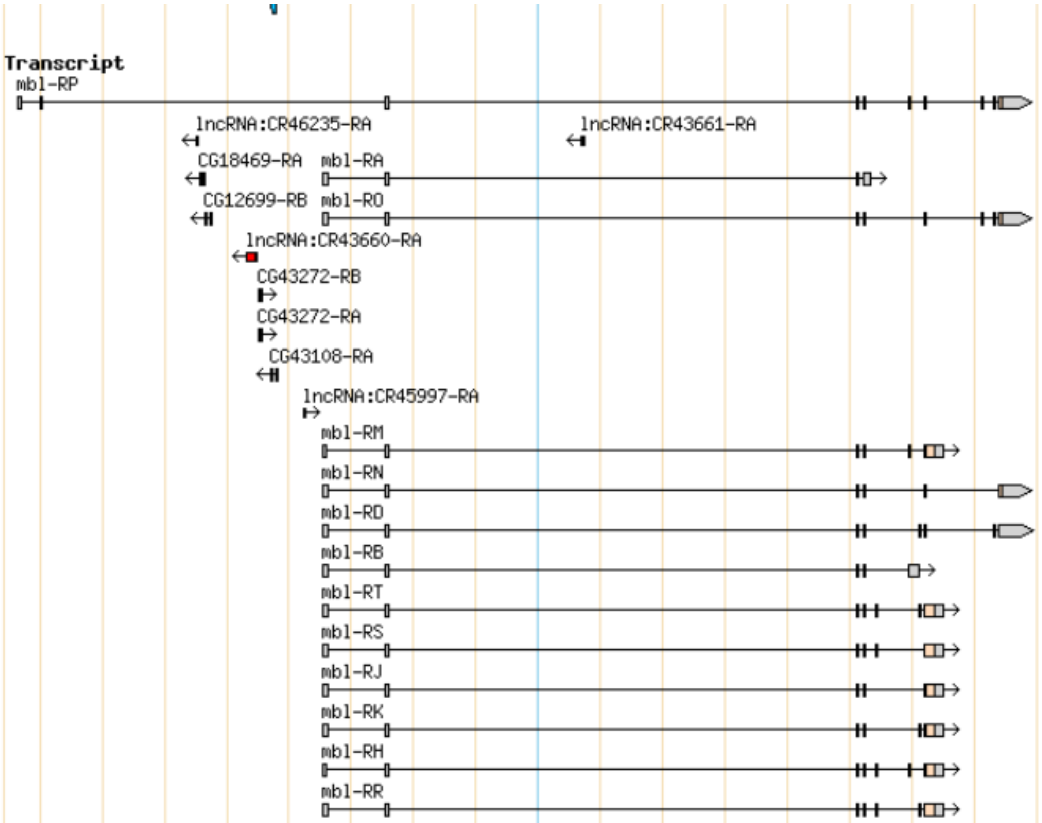

Figure 1. A screenshot from the FlyBase database showing the number of transcripts generated from the *mbl* in *Drosophila*

and unique polypeptide sequences are identified that align with *mbl* locus (Fig1). However, so far, only 4 isoforms out of 13, mblA, mblB, mblC and mblD have been extensively characterized for their expression and molecular functions. These isoforms are functionally distinct and differentially expressed through the life cycle of *Drosophila* and known to have varied ability to rescue embryonic lethality of *mbl* mutant flies(Vicente et al., 2007). In *Drosophila*, α-actinin pre-mRNA undergo developmentally regulated alternative splicing to generate A, B, C isoforms at different developmental stages. This is facilitated by the MBL binding consensus sequences in *α-actinin* pre-mRNA(Vicente et al., 2007). The *muscleblind*-dependent alternative splicing of α-actinin has shown to be misregulated in Myotonic dystrophy models. The four characterized splice variants of *mbl* share their N-termini with either one as in mblD or two as in mblA, mblB, mblC Cys3His zinc- finger motifs, but have alternative carboxy-terminal ends(Irion, 2012). It has been shown that the alternative splicing of *up* (*troponin-T*), *WupA* (*troponin-I*) the components of troponin complex and *Zasp-52* the muscle-specific genes are shown to be misregulated in *mbl* mutants. Besides this, *kkv (krotzkopf-verkehrt, coding for* Chitin Synthase 1*) and cora (coracle,* coding for the Drosophila homolog of Protein 4.1) that are not muscle specific genes are shown to be MBL dependent for alternative splicing and mis-spliced in *mbl* mutants. Importantly, out of other isoforms mbl-C seems to be evolutionarily conserved with high rescuing ability of the *mbl* mutant phenotypes. It has been shown that conserved FKRP motif which is important for SUMolyation, a post-translation modification that influences the subcellular localization of a protein is present in MBL-C isoform. Mutation of FKRP motif in MBL-C, reduced its nuclear localization and enhanced cell-death inducing activity of MBL-C(Vicente-Crespo et al., 2008). This feature of MBL-C makes it functionally distinctive from MBL-A although both share zinc-finger motifs. Recently, deterministic splicing activity of *muscleblind* has also been reported. It has been shown that MBL represses the selection of Dscam2 10A exon and necessary for the inclusion of Dscam2 10B exon in specific cell types. It has also been shown that the expression of *mbl* is cell type specific and its ectopic expression in *mbl* negative cells leads to inclusion of Dscam2 10B exon(Li & Millard, 2018).

On the other hand, the role of MBL is also involved in biogenesis of circular RNAs. circRNAs are mostly the class of non-coding RNAs whose functions remain unknown. Circular RNAs are formed co-transcriptionally as a result of back-splicing either facilitated by specific RBPs binding to the flanking introns or by the inverted complementary sequences in the flanking introns bringing the exons to form exonic circRNAs as illustrated in the Fig2 (Patop & Kadener, 2018).

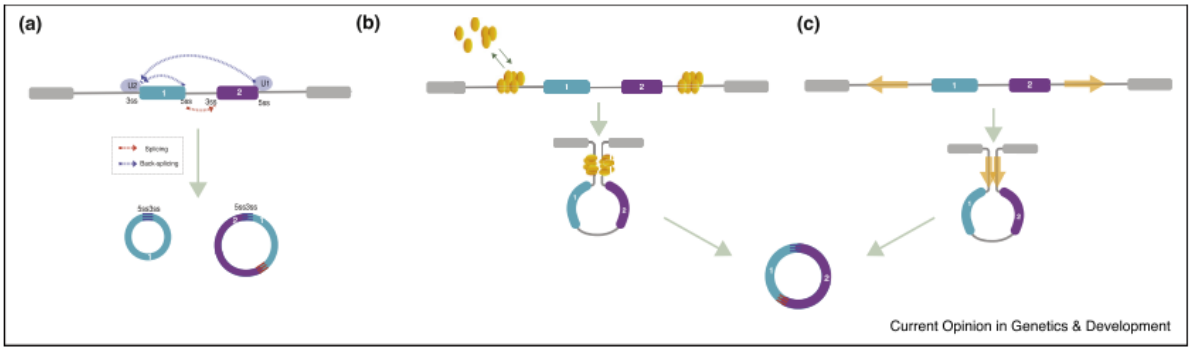

Figure ii: General mechanism of circRNA biogenesis. (a) Regular splicing (red arrows) and back splicing (blue arrows) give origin to mRNA and circRNA, respectively. Both processes are co-transcriptional and can compete between each other. circRNAs have a specific junction, unique for this molecule (blue dashes). CircRNAs can also contain exons which are joined by regular splicing and have a junction shared with the linear form (red dashes). (b) Back splicing can be induced by proteins that bind to specific regions in the flanking introns and help to get the circularizing exons together. (c) Exon circularization can also be induced by inverted complementary sequences (yellow arrows) in the flanking introns that stabilize and bring together the exons involved in back splicing. (Taken from Patop & Kadener, 2018)

It has been shown that the 2nd exon of the *mbl* locus is circularized to form the most abundant circRNA, circMbl in *Drosophila.* The circMbl and its flanking introns contain conserved MBL binding sites that are strongly bound by MBL protein. CircMbl biogenesis can be affected by modulation of MBL protein levels and dependent of MBL binding sites. It has also been shown that the overexpression of MBL-A and C isoforms in *Drosophila* S2 cells enhances the circMbl production(Ashwal-Fluss et al., 2014). Ribosome foot-printing assay has revealed that the circMbl is associated with ribosomes and translates into protein in synaptosomes via cap-independent translation(Pamudurti et al., 2017). Based on these interesting findings and it is being abundantly expressed in flies, it is important to understand the molecular interactions required for the biogenesis of circMbl *in-vivo*. This thesis attempts to provide the initial findings on the effect of different MBL isoforms in circMbl biogenesis in a sex-specific manner and the expression profile of circMbl in Myotonic Dystrophy models in *Drosophila.*

Materials and Methods:

Fly Stocks:

w*; Actin5c-Gal4/CyO (that was the lab stock) was used as a Gal4 driver for the ubiquitous expression. Elav-Gal4 on X was used as a Gal4 driver for PAN-neuronal expression.

UAS-mbl-A (on 3rd), UAS-mbl-B (on 3rd), UAS-mbl-C (on 3rd) and UAS-iCTG480 (on 3rd) fly lines were generously gifted by Prof. Ruben Artero, Dept. of Genetics, University of Valencia, Spain.

UAS-CTG240 (on 3rd), UAS-CTG480 (on 3rd), UAS-CTG600 (on 3rd), UAS-CTG960(on 3rd), fly lines were generously gifted by Prof. Krzysztof Jagla, University of Clermont-Ferrand, France

Primers used for RT-PCR:

mblA-Fwd	5'-CAGACACCGAAATACTCTCTACAAACA-3'
mblA-Rev	5'-AAAATCAGGAGTAAACAAATACACGTAGAC-3'
mblB-Fwd	5'-CACACATCCAGATATGCTACTTACCA-3'
mblB-Rev	5'-TGAGCGATTTCGATTGATTTTG-3'
mblC-Fwd	5'-CAGCAAACACACATCACCTACCA-3'
mblC-Rev	5'-CTATCGAGCAGGAGGATGAAGAG-3'
circMBL_F	5'-AGGACACCGAATGCAAGTTC-3'
circMBL_R	5'-AAACGCAGCTGTTAATTTTTG-3'
CUG_F	5'-AAGGAACCTTACTTCTGTGG-3'
CUG_R	5'-TCATCAGTTCCATAGGTTGG-3'
circMbl2345_F	5'-CATGTTTCCACTGATAATGTAAACTC-3'
circMbl2345_R	5'-GATTCAAATCGAACGCTTATTC-3'

Methods:

***mbl* isoforms overexpression crosses and collection of fly heads:**

The crosses were setup at 25°C with 10 virgins of Gal4 stock crossed with 7-8 males of UAS stocks or w^{1118}. Parents are transferred into fresh media bottle once every three days for two times. The progeny (30-40 per biological replica) of age between 2-3 days were collected into 15ml falcon tubes and frozen in -80°C freezer for collecting the heads the next day using metal sieves.

Cell culture and transfections

Drosophila S2 cells were maintained in 10% fetal bovine serum (Invitrogen) Schneider's insect medium (GenClone). Cells were seeded in a six-well plate. Transfection was performed at 60%–80% confluence according to company recommendations: 6µl of TransIT 2020transfection reagent (Mirus Bio, MIR 5400A) and 2µg of total DNA. cells were collected 48h after the transfection.

Analysis of gene expression by real-time PCR

Total RNA was prepared from adult fly heads (30 heads per sample) using Trizol reagent (Sigma) according to the manufacturer's protocol. cDNA derived from this RNA (using iScript Bio-Rad) was utilized as a template for quantitative real-time PCR performed with the C1000 Thermal Cycler Bio-Rad. The PCR mixture contained Taq polymerase (SYBR green Bio-Rad). Cycling parameters were 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 30 s. Fluorescence intensities were plotted versus the number of cycles by using an algorithm provided by the manufacturer. mRNA levels were quantified using a calibration curve based upon dilution of concentrated cDNA. mRNA values from heads were normalized to that from ribosomal proteins 49 (Rp49) and/or 28s.

Nuclear/cytoplasmic fractionation

Fly heads were homogenized in a Dounce homogenizer, in the following buffer: 10 mM Hepes pH 7.5, 10 mM KCl, 0.8 M Sucrose, 1 mM EDTA, 0.5 mM DTT, supplemented by protease-inhibitor cocktail (mini complete, Roche) and phosphatase inhibitors. After homogenization, the homogenate was filtered through a column polymer bed support (Bio-Rad unfilled Bio-spin

Column 4 minutes 1000 g 4°C) to remove the cuticle. The filtrate was then centrifuged (600 g, 10 minutes 4°C) and the pelleted cell extract were then subjected to nuclear cytoplasmic fractionation. In case of S2 cells the cells were centrifuged at 600g, for 10 minutes 4°C. To prepare the cytoplasmic fraction, the cell pellets were re-suspended in cytoplasmic buffer (10 mM Tris HCl pH 8.0, 10 mM KCl, supplemented by protease inhibitor cocktail (mini complete, Roche) and phosphatase inhibitors). Cells were allowed to swell for 15 minutes, and then NP-40 was added to 0.4%, followed by centrifugation (3500 g, 3 minutes 4°C). The supernatant contained the soluble cytoplasmic fraction. The pellets were washed once more with the cytoplasmic buffer before proceeding to nuclear fractionation. For the preparation of the nuclear fraction, the remaining cell pellet was re-suspended in high-salt buffer (50 mM Tris pH 8.0, 5 mM EDTA, 400 mM NaCl, 1% NP-40, 1% Sodium deoxycholate, and 0.025% sodium dodecyl sulfate (SDS), 1 mM DTT supplemented by protease inhibitor cocktail (mini complete, Roche) and phosphatase inhibitors). The nuclear pellet was hard vortex for 30 min at 4°C and was then centrifuged (1 minute 4°C max speed). The supernatant, which contains the nuclear fraction was saved. Both fractions were re-suspended in protein sample buffer, heated 5 minutes 95°C

Generation of *mbl* isoform specific shRNA lines

mblA exon4-3'UTR

ShRNAi line1: GAAGTCAATTTAACGCGATTT

(F)Ctagcagt GAAGTCAATTTAACGCGATTTtagttatattcaagcataAAATCGCGTTAAATTGACTTCgcg
(R)aattcgcGAAGTCAATTTAACGCGATTTtatgcttgaatataactaAAATCGCGTTAAATTGACTTCactg

ShRNAi line2: CTATGTATACGAATATTGCGA

(F)CtagcagtCTATGTATACGAATATTGCGAtagttatattcaagcataTCGCAATATTCGTATACATAGgcg
(R)aattcgcCTATGTATACGAATATTGCGAtatgcttgaatataactaTCGCAATATTCGTATACATAGactg

GCAACAAACCCCTATCTGACCGGCATTCCGGCCAACTCGTACAGTCCGTACTACACAA
CGGGACACCTGGTGCCCGCCTTGCTGGGTCCGGATCCCGTGACCTCCCAACTGGGAC
CCGTGGTGCCCCAAACAGTGCAGGTGGCACAGCAGAAAATACCACGTTCCGACAGATT
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TCCAATGAAGTCAATTTAACGCGATTTTATTTACGCTTACTTTGTGTTTATTGATTTAACA
AAAGTTTTTTACGTTCCAATGTTTCCTTTCGTGGCATGTCTTTATGTATTTATATTTCTAAT
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CTGATTTTGAATAATCCTTGCATTCTCGTTGTTCCTTTTTGTTATGTTTGTGCTACGTGTT
CCTCTTGTGTTTTCTCGTACTcaaaagtgcaccaacttaaaaaatgcaaattgaaaactaatcggcatgaagcaa
aaataaaaaccacacccaaaaataaaccaaTGCAATACCAATTCCAAGGGGCAAGTACAATAGCAA
GaaaaaaaaagcataatcaaaaaaatcctaacacaaataaaaccaaaatttgaaatGCATCTGTATCGGCTCTA
ACCACTAAAC

mblB Exon5 and 3'UTR

Sh line 1: GCGTACGAAGAGATCCCACGA

(F)CtagcagtGCGTACGAAGAGATCCCACGAtagttatattcaagcataTCGTGGGATCTCTTCGTACGCgcg
(R)aattcgcGCGTACGAAGAGATCCCACGAtatgcttgaatataactaTCGTGGGATCTCTTCGTACGCactg

Sh line 2: ATTTACGACATTAGTTTGTTA

(F) CtagcagtATTTACGACATTAGTTTGTTAtagttatattcaagcataTAACAAACTAATGTCGTAAATgcg
(R) aattcgcATTTACGACATTAGTTTGTTA tatgcttgaatataactaTAACAAACTAATGTCGTAAATactg

acctcacctcttgcggcacaccatcaccagcaacaacaacaattgcaacaccagctgaacaacatcaacaacaacaacaac
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mbIC Exon6-Exon7

sh line 1: ACGTGCCAGTCTCATTTACTG

(F)CtagcagtCCAGTCTCATTTACTGGACACtagttatattcaagcataGTGTCCAGTAAATGAGACTGGgcg
(R) aattcgcCCAGTCTCATTTACTGGACACtatgcttgaatataactaGTGTCCAGTAAATGAGACTGGactg

sh line2: GTGCCAGTCTCATTTACTGGA

(F)CtagcagtGTGCCAGTCTCATTTACTGGAtagttatattcaagcataTCCAGTAAATGAGACTGGCACgcg
(R) aattcgcGTGCCAGTCTCATTTACTGGAtatgcttgaatataactaTCCAGTAAATGAGACTGGCACactg

CAATTCTCTGGCATGGTACCGTTCAAACGTCCAGCTGCCGAAAAGTCTGGCATTCCAGT
TTATCAGCCCGGTGCGACCGCCTATCAGCAGCTAATGCAGCCCTACGTGCCAGTCTCA
TTTACTGGACACCCTCCCGGTGTGCCAAGATTTTAATAGATCTATGTGCAGTCGGTTAA
ATTGTAGATTCGTTCATTTAACTGAAG

mbID exon3

Sh line1: CAGCAAACAACTTGAAATGTA

(F)CtagcagtCAGCAAACAACTTGAAATGTAtagttatattcaagcataTACATTTCAAGTTGTTTGCTGgcg
(R) aattcgcCAGCAAACAACTTGAAATGTAtatgcttgaatataactaTACATTTCAAGTTGTTTGCTGactg

Sh line2: TATGCCTTTGCCCCGAATGTA

(F)CtagcagtTATGCCTTTGCCCCGAATGTAtagttatattcaagcataTACATTCGGGGCAAAGGCATAAgcg
(R) aattcgcTATGCCTTTGCCCCGAATGTAtatgcttgaatataactaTACATTCGGGGCAAAGGCATAactg

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ATTTCATTCGAAATTGAAGTCGAATGGCGAATTCAATTATAAATATGCTTACATTTGCATC
TGTCAATTTAGTTGCAAATGTTGTGTTGCCGATGGGATAGGCaaaa

Results:

***In-vivo* interaction of circMbl and MBL in neurons:**

Biogenesis of circMbl from the 2nd exon of *muscleblind* (*mbl*) locus is facilitated by MBL protein(Ashwal-Fluss et al., 2014). It has been shown, in *Drosophila* S2 cells, that the overexpression of MBL isoforms A and C enhances the expression of circMbl. However, MBL-C is the only isoform that is expressed in the adults(Vicente et al., 2007). Based on this, I performed overexpression studies *in-vivo* by using UAS-Gal4 system. I crossed the flies individually with transgenes containing UAS-mbl-A, UAS-mbl-B and UAS-mbl-C(Juni & Yamamoto, 2009) to Actin5C-Gal4 flies using w^{1118} as a control.

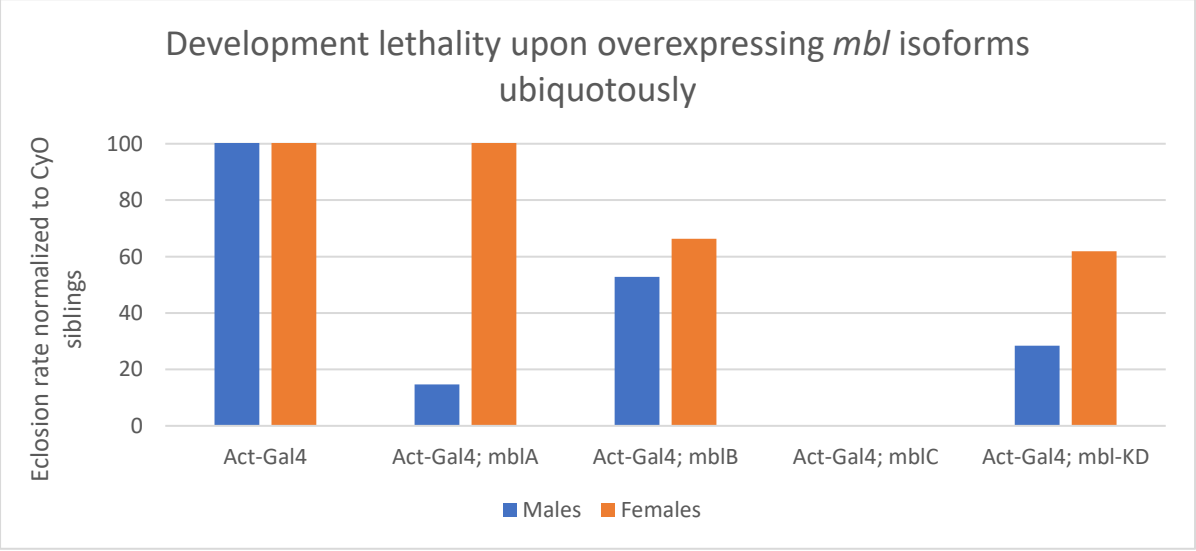
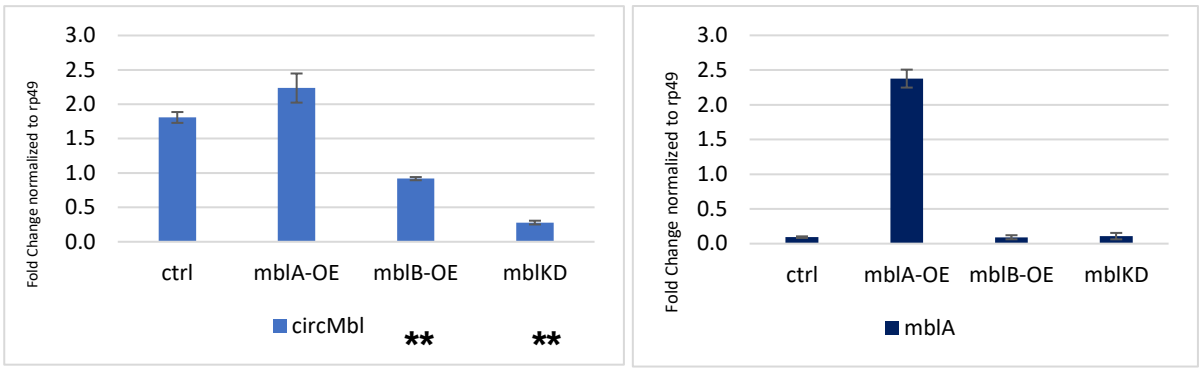


Figure1: Developmental lethality caused by the overexpression of *mbl* isoforms: mblA-OE causes male specific lethality similar to mbl-KD by RNAi. MblB-OE causes reduction in the eclosion rate. MblC-OE causes complete developmental lethality.

Ubiquitous overexpression of mbl-A causes male specific lethality with a few escapers while overexpression of mbl-C causes complete developmental lethality. But the overexpression of mbl-B has not caused any significant change in male to female ratio but the eclosion rate was less when compared to controls. As predicted, mbl-KD also caused male lethality and reduction in female eclosion rate.



I examined the circMbl levels in fly heads upon overexpression of *mbl* isoforms by RT-qPCR. The circMbl levels are altered in fly heads when MBL isoforms are overexpressed. As previously shown by the

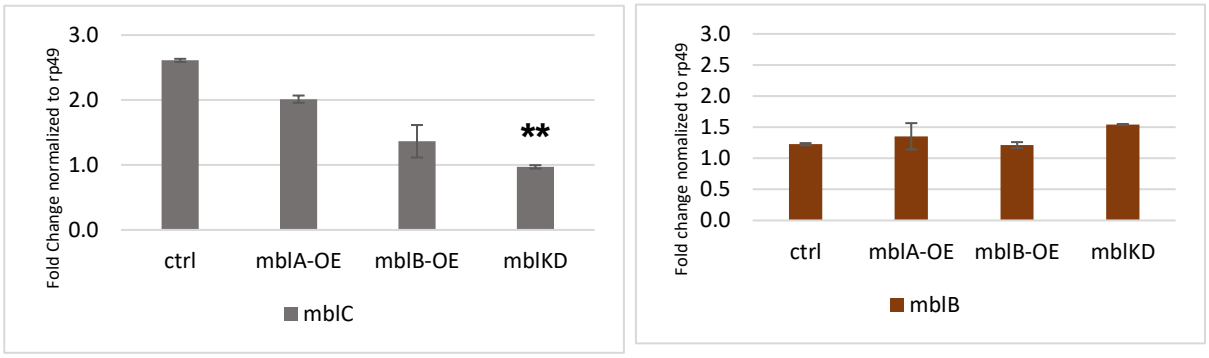


Figure 2: A. Quantification of circMbl expression in fly heads upon overexpression of MBL isoforms by RT-qPCR. **B, C, D.** RT-qPCR quantification of mbl-C, mbl-A and mbl-B respectively in fly heads of ctrl, mbl-A-OE, mbl-B-OE, mbl-KD. P-values are calculated using two-tailed Student T. Test with equal variance. ** p-Values<0.01

Kadener lab, that mbl-A upregulates the circMbl production *in vitro*. As shown in the Fig2, the *in-vivo* overexpression of mbl-A also increases the production of circMbl. However, I observed the decrease in circMbl levels upon overexpression of mbl-B too, although mbl-B transcript levels seem unaltered. To investigate whether other isoforms are altered due to the overexpression of mbl-B, causing reduction in circMbl levels upon overexpression of mbl-B, I wanted to look at the expression of either mblA or mblC, which are shown to regulate the circularization of circMbl. It came to my surprise that the overexpression of mbl-B causes reduction in mbl-C expression which might be affecting the biogenesis of circMbl. From the previous literature it is known that the mbl-A does not express in the adult stage and the same is observed in Fig2C and the expression is also not altered upon mbl-B overexpression. However, mbl-C that is known to be more expressed in adult stage is altered upon over expression of other isoforms. Importantly the overexpression of mbl-B decreases the

expression of mbl-C similar to the level of *mbl* knockdown by RNAi which hints that the reduction of circMbl in mbl-B overexpression may be caused by the reduction in mbl-C isoforms. A further validation at protein levels by using techniques like western blot might support this argument.

As the ubiquitous overexpression of mbl-C caused developmental lethality, I wanted to minimize this effect by using a tissue specific Gal4 driver, to validate the regulation of circMbl by mbl-C isoform. To do so, I overexpressed *mbl* isoforms in neurons using a PAN-neuronal driver, *elav*-Gal4. The overexpression of mbl-A, mbl-B and mbl-C in CNS haven't caused any developmental lethality. As the overexpression of mbl-A ubiquitously caused the male specific lethality. Similarly, ubiquitous knockdown of circMbl has also caused partial male lethality(Pamudurti et al., 2018). I suspected that the MBL isoforms might have sex specific

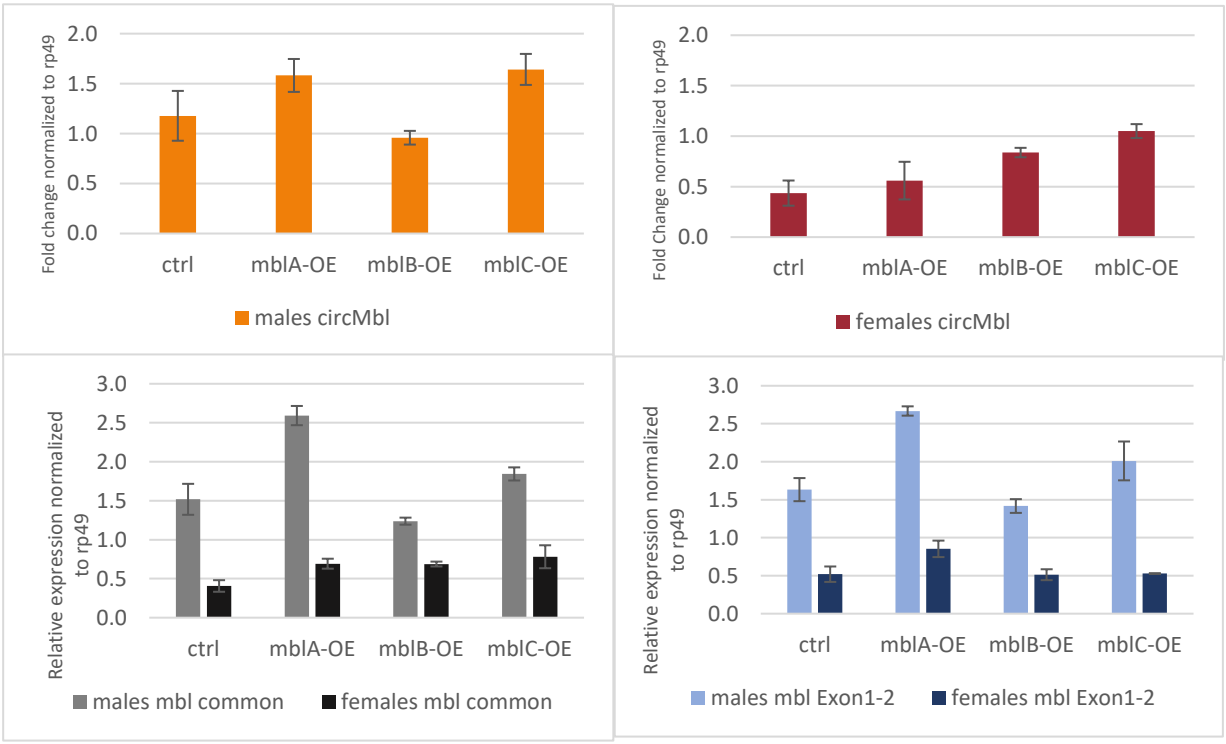


Figure 3: A. Expression levels of circMbl in males upon overexpression of mbl isoforms PAN-neuronally **B**. Expression levels of circMbl in females upon overexpression of mbl isoforms PAN-neuronally. **C & D.** Quantification of *mbl* linear transcripts using two different sets of primers

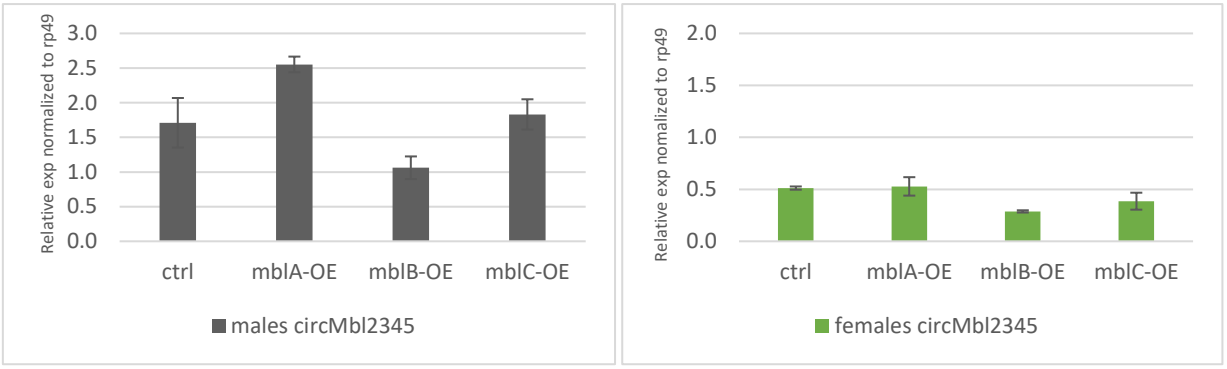


Figure 4: A&B**.** Expression levels of circMbl2(with exons2345) in males and females upon overexpression of circMbl

roles. Hence, I quantified the expression of various *mbl* transcripts including its circular RNAs by RT-qPCR. Initially, I used a common primer pair that amplifies the exons that are common to all isoforms and a primer pair that amplifies exon1&2 excluding circular isoforms. As shown in the figure 3C&3D, the expression of *mbl* transcripts seems to be more in males than females.

As observed when the *mbl* isoforms overexpressed ubiquitously, the overexpression of mbl isoforms pan-neuronally also altered the circMbl expression levels. Overexpression of mbl-A increases the circMbl and circMbl2(circular RNA formed including exons 2-5) expression levels in males (Fig3A & 4A), whereas overexpression of mbl-B reduces the expression of circMbl in males and increases in females (Fig3B) and increases circMbl2 expression both in males and females (Fig4A&B). Overexpression of mbl-C increases the expression of circMbl in males and females (Fig3A& 3B) whereas circMbl2 levels are not significantly altered (Fig4A&4B).

Generation of RNAi reagents to knockdown specific isoforms of *mbl*:

To validate the effect of overexpression of each isoform on circMbl biogenesis, I planned to generate shRNA lines to knockdown the specific *mbl* isoforms. To do so, I chose a 21nt sequence from an exon or combination of exons that is unique to the targeted isoform (please see the materials and methods section for the sequences). Those 21nt sequences are included into the miR-1 pre-miRNA template to clone into a pVALIUM20 vector used to

generated RNAi lines under TRiP project. The plasmids were cloned, sequenced and sent for microinjection for generating transgenic lines.

Characterization of CircMbl expression in Muscular Dystrophy1 disease models:

Myotonic Dystrophy type-1 is a neuromuscular disorder characterized by symptoms including myotonia and muscle degeneration. In DM1 patients, RNA toxicity caused by the CTG repeats in the 3' UTR of the *DMPK* gene form nuclear foci and sequester the MBNL1 protein a splicing factor. Mbl is one of the proteins involved in the etiology of MD1.

To examine whether the expression of CTG repeats and sequestration of MBL protein into the nucleus would also alters the production of circMbl, I overexpressed the increasing number of CTG repeats say 240, 480 and 960 and iCTG480(Ruben Artero's fly stock) under the control of UAS driven by using a neuronal driver, *elav*-Gal4 and w^{1118} crossed to elav-Gal4 is taken as a control. Quantification of circMbl expression by qRT-PCR from the RNA isolated from the

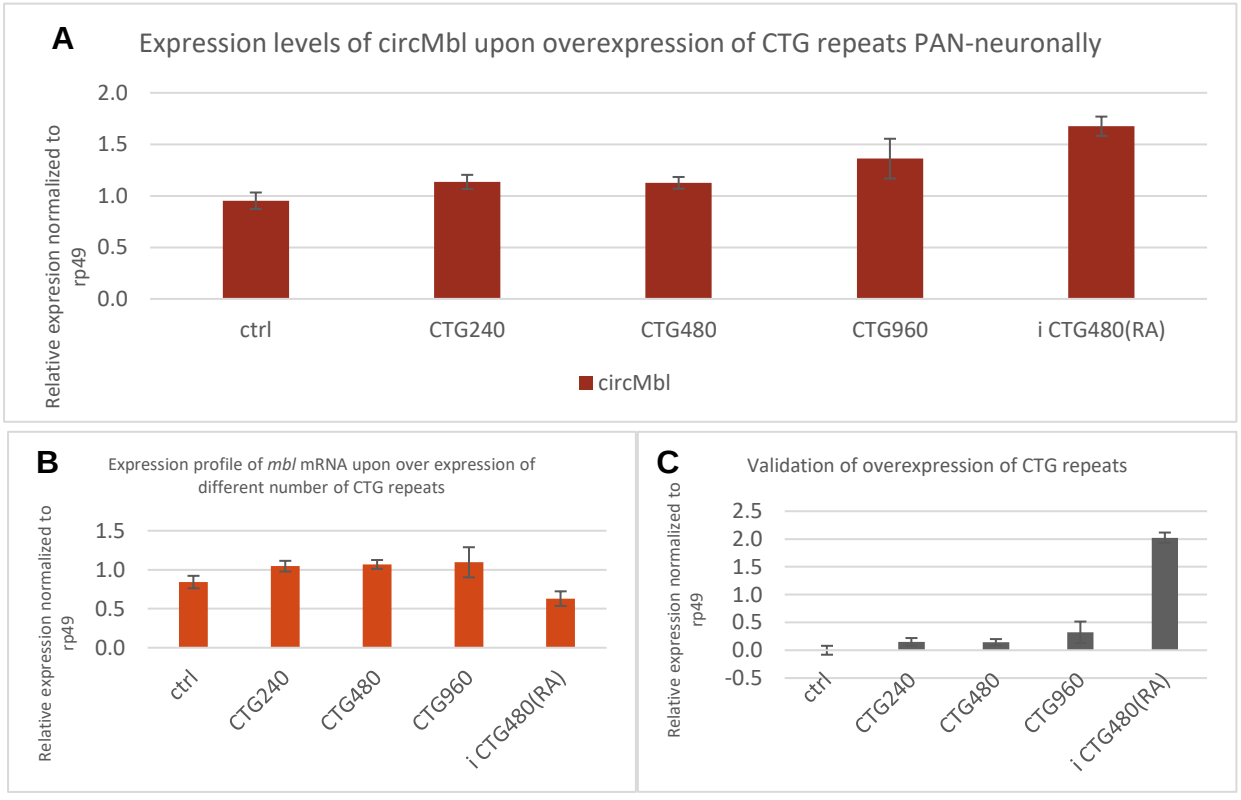


Figure 5: Quantification of circMbl expression in Myotonic Dystrophy models. A. Quantification of circMbl from the RNA isolated from fly heads with different number of CTG repeats overexpressed.

adult heads has revealed that the overexpression of CTG repeats in neurons alters the circMbl

levels. As shown in the Fig5A, overexpression of 240 and 480 CTG repeats haven't altered circMbl levels where as the overexpression of 960 CTG and iCTG480(RA) have significantly increased the expression of circMbl in adult fly heads. Further, to validate whether the effect is due to the overexpression of CTG repeats, I quantified the overexpression of CTG repeats. As shown in the Fig5C, 240 and 480 CTG repeats were expressed modestly while 960CTG repeats was more expressed. However, iCTG480 was expressed 10 folds higher than 240 CTG repeats. I also wanted to check the *mbl* linear mRNA levels, hence I used a primer pair that amplifies a part of transcript from the exons 1 and 2 which are common exons for all linear transcripts of *mbl* gene. Although overexpression of 240, 480, 960 repeats have not significantly altered the levels of mbl mRNA, overexpression of iCTG480 has reduced their levels (Fig5B).

The above results demonstrate that the increase in the circMbl levels in the neurons might be due to the sequestration of MBL protein by the overexpressed CTG repeats. In other words, there should be more amount of protein in nucleus compared to cytoplasm because of the

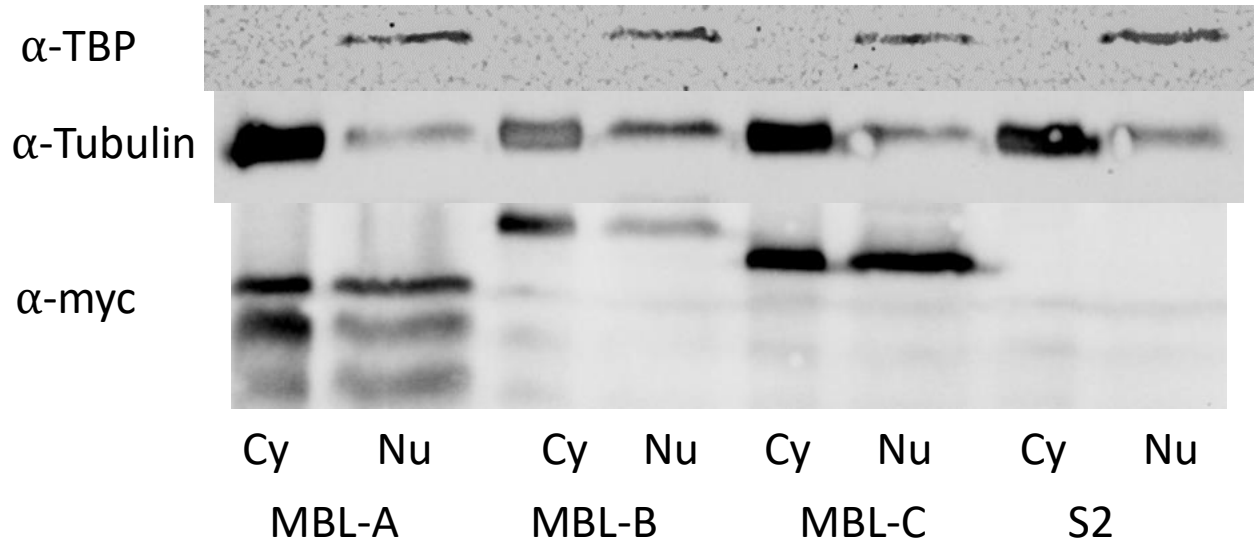


Figure 6: A Western Blot with anti-*myc* staining of the protein samples from cytoplasmic and nuclear fractions. Anti-Tub and anti-TBP were used as controls for cytoplasmic and nuclear fractions respectively and validate for any cross contamination.

sequestration effect caused by CTG repeats. I thought the best way to validate this, is by isolating cytoplasm-nuclear fractionations and performing antibody staining against MBL on a western blot. To optimize the fractionation protocol and better understand the subcellular

localization of MBL isoforms, I overexpressed the myc-tagged MBL isoforms mbl-A, mbl-B and mbl-C in S2 cells co-transfected with Actin-Gal4 plasmid.

From this experiment, I observed that MBL-A and MBL-C localized in both cytoplasm and nucleus while MBL-B was predominantly present in cytoplasm as shown in the figure 6.

After optimizing the fractionation protocol, I wanted to extract cytoplasmic and nuclear fractions from the fly heads in which the CTG repeats are expressed and perform α-*mbl* staining to examine the MBL localization. For this purpose, I chose the fly heads from the progeny from iCTG480 crossed to *elav*-Gal4 along with Gal4 controls. Based on the well characterized molecular interaction between the CTG repeats and MBL proteins in forming nuclear foci upon the expression of CTG repeats, I predicted that MBL protein should localize

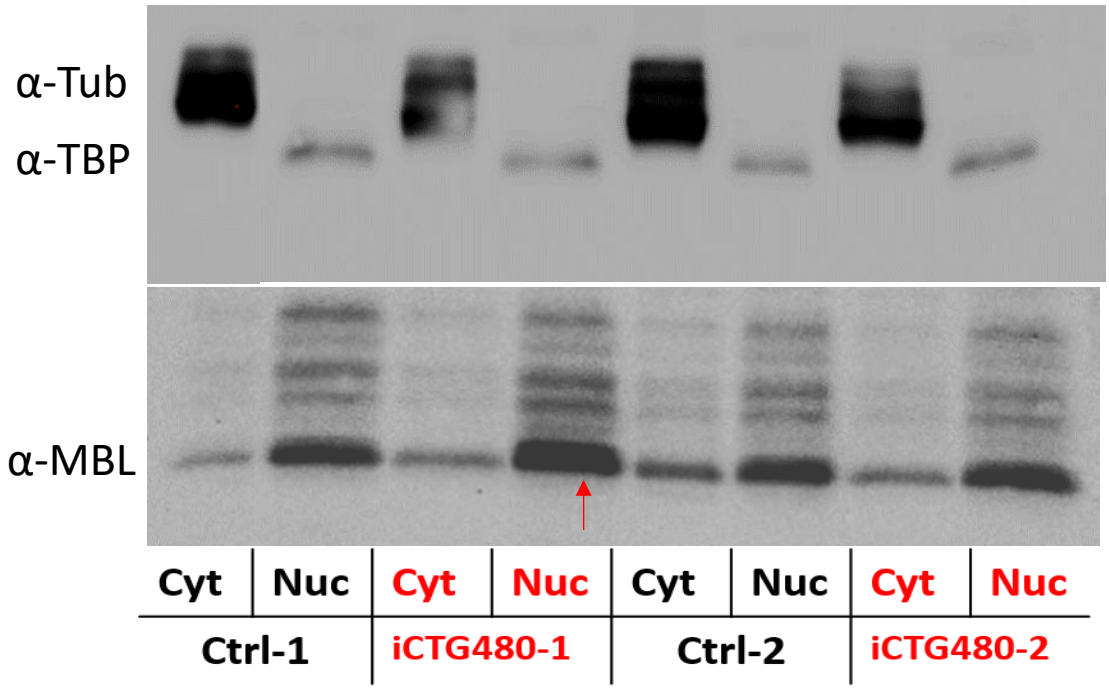


Figure 7: Western blot of anti-MBL on the protein samples from cytoplasmic and nuclear fractions from fly heads. MBL protein of ~27kD size present in nuclear fractions more than the cytoplasmic fractions in iCTG480 samples. Ctrl: Elav-Gal4 (X) w^{1118} and iCTG480: Elav-Gal4 (X) UAS-iCTG480

more to the nucleus. In other words, I should see denser bands of MBL in nuclear fractions of iCTG480 samples when compared to the control samples that lack CTG repeats. In consistent with the prediction, as shown in the Fig 7, the amount of MBL protein localized in the iCTG480-1 nuclear fractions on the western blot looks more(denser) than the control fractions due to

sequestration of MBL protein by the expressed CTG repeats. I have also stained the protein extracts from the nuclear and cytoplasmic fractions with anti-Tub and anti-TBP respectively, to validate the purity of subcellular fractions and to know if any cross contamination has occurred. Further, to examine the distribution of RNA in these cellular extracts, I extracted the RNA and quantified the expression of circMbl from the fractions. As predicted, I observed more circMbl in the cytoplasmic than in the nuclear fractions, in both controls and iCTG480 repeats. Interestingly, I observed a slight increase in the circMbl expression levels within the nuclear fractions when compared to the controls (fig 8). I also looked at the expression of CTG repeats in both the fractions, as obvious, there are significantly higher expression of CTG repeats in iCTG480 fractions. I hypothesized that there should be change in the pre-mbl levels due to the presence of more MBL protein in the nucleus facilitating circMbl production. However, I could not detect the changes in the levels pre-mbl with the primer set landing on 2nd exon and flanking intron.

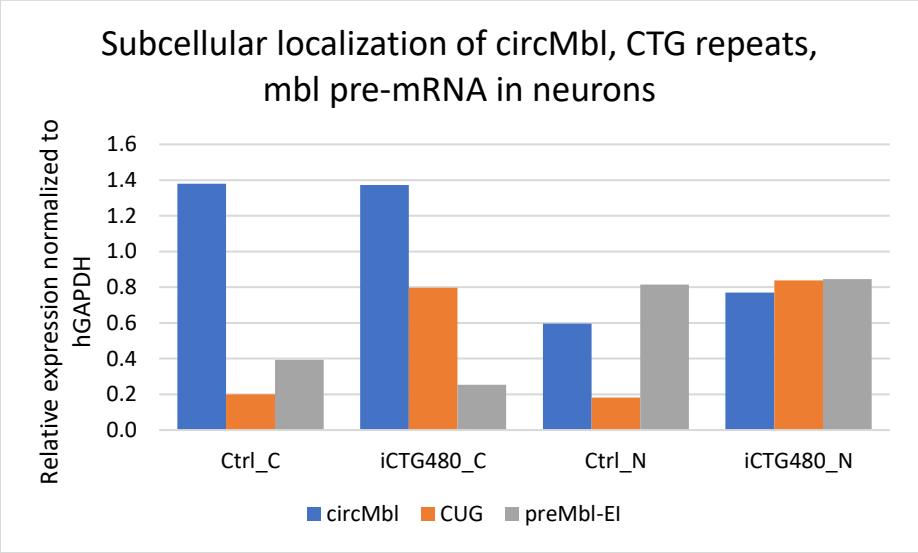


Figure 8: Quantification of RNA expression from the cytoplasmic and nuclear fractions from the heads of flies with CTG480 repeats and controls

Discussion:

Circular RNAs are re-discovered class of non-coding RNAs, most of whose functions are not known. CircRNAs are highly abundant in neuronal tissues of human and mouse brain and they are detected in *Drosophila* neuronal tissues as well(Rybak-Wolf et al., 2015) and they are evolutionarily conserved. Despite their existence across the animal kingdom, the molecular functions of these RNA species are not studied well. MUSCLEBLIND family proteins are also evolutionary conserved RNA binding proteins that are extensively studied for its role as a splicing factor in a context dependent way (Oddo, Saxena, McConnell, Berglund, & Wang, 2016). The splicing activity of MBNL proteins are reported to be misregulated in myotonic dystrophy patients due to the expression of CTG repeats from the 3' UTR of the mutant *DMPK* gene. These CTG repeats sequester the MBL protein to form nuclear foci disrupting its normal functions which has been shown in mammalian tissues and disease models in Drosophila(Ho, 2005; Philips, 1998; Picchio, Plantie, Renaud, Poovthumkadavil, & Jagla, 2013). In *Drosophila,* the gene *muscleblind* an ortholog of human MBNL1 has distinct regulatory functions in muscle and neuronal differentiation. A circular RNA, circMbl produced form the *mbl* locus has shown to be affected by the expression of *mbl* isoforms *in-vitro*. This thesis demonstrates the relationship between the MBL protein and circMbl *in-vivo* and characterizes the circMbl expression in DM1 fly models. The data in this thesis suggest that ubiquitous overexpression of mbl-C during the development of *Drosophila* causes lethality while mbl-A causes male specific lethality with few escapers. Mbl-A-OE also enhances the circMbl expression. However, overexpression of mbl-B causes reduction in the eclosion rates when compared to the controls, this observation seems to be consistent with the previous reports(Vicente et al., 2007). Moreover, it causes reduction in the levels of circMbl. This might be due to the reduced levels of mbl-C upon overexpression of mbl-B because these isoforms

are differentially expressed through the development with distinct functions. Additionally, quantification of RNA by RT-qPCR that is isolated separately from equal number of males' and females' heads revealed that the circMbl levels are differently regulated by *mbl* isoforms in as sex specific manner. In addition to this it is also evident that the transcription of *mbl* locus is more in males than in females. Because the circMbl and other linear isoforms are expressed two folds higher in males than females. This suggest that not only *mbl* but also circMbl might also have sex-specific roles in the development of *Drosophila*. As recently it has been shown that the circMbl can also be translated into a short protein in a cap-independent way regulated by FOXO or starvation, its molecular function is not understood yet. On the other hand, ubiquitous knockdown of circMbl through the development causes male partial lethality and wings-up phenotype in males(Pamudurti et al., 2018). In this background, it is worth investigating whether the circMbl protein forms a dimer and acts as substitute for MBL function or work in synergy with MBL linear isoforms during the metamorphosis. DM1 disease models in *Drosophila* have been extensively used to characterize how the MBL alternative splicing function is disrupted but there have been no reports showing this effect on circMbl biogenesis. In this thesis we have shown that circMbl biogenesis is influenced by the overexpression of CTG repeats enhancing the circMbl expression in fly heads. This change can be attributed to the sequestration of MBL by CTG repeats to the nucleus which might result in increased binding to *mbl* pre-mRNA and facilitating the excessive circularization of 2nd exon forming circMbl. We have also proven this hypothesis by performing antibody staining against MBL on the western blots from the cellular fractions showing the increased localization of MBL protein in nucleus than cytoplasm when compared to the controls. In consistent with the previously reported subcellular localization of *mbl* isoforms by antibody staining, our western blot assays from the cellular fractions of S2 cells also revealed that mbl-A and mbl-C present in both cytosolic and nuclear fractions while mbl-B(~33kD) is only present in nuclear fractions. Also, it has become evident that mbl-B which localizes in the nucleus has been seen only in nuclear fractions in both controls and DM1 models whereas the mbl-C(~27kD) which is present in the both cytoplasm and nucleus also seen in both fractions but with increased amount in the

nuclear fractions in CTG repeats expressed cells. Also, the circMbl levels seems to be increased in CTG repeats nuclear fractions than the controls. Taking together, this data suggests that not only MBL dependent alternative splicing of linear transcripts is affected by the disruptive CTG repeats in DM1 models, but also circMbl biogenesis. However, the molecular mechanisms underlying this, need to be deciphered. It is also required to understand how MBL protein while sequestered by the CTG repeats, can also facilitate the circularization of 2nd exon to form circMbl. This should be possible with two different binding domains for CTG repeats and YGCY motifs present in MBL protein, which need to be validated by the techniques like PAR-CLIP from these fractions. Also, there might be an auto regulatory feedback loop that triggers the circularization of circMbl when there is more MBL protein present in the nucleus instead of splicing linear transcripts. A similar auto regulatory loop has been reported in the mammalian tissues where MBNL1 interacts of exon1 of MBNL pre-mRNA to control steady state levels of MBNL1(Konieczny, Stepniak-Konieczna, Taylor, Sznajder, & Sobczak, 2017)

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