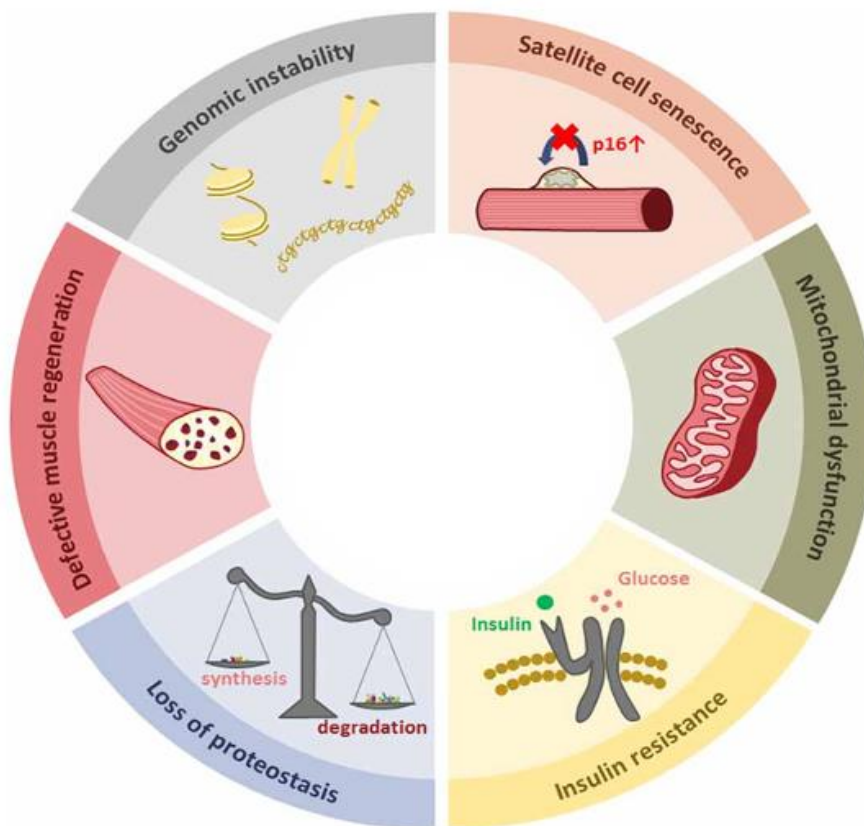




UNDERSTANDING THE PATHOGENESIS AND POTENTIAL THERAPEUTICS OF MYOTONIC DYSTROPHY



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ABSTRACT

Myotonic dystrophy is the most common type of muscular dystrophy, a genetic condition that is known for causing the muscles to progressively weaken. Other clinical symptoms include prolonged muscle contractions, cataract, diabetes, cardiac defects, respiration issues, and more. The clinical variability, multi-system involvement and increasing severity of symptoms with generations of the disease makes it complex to cure. There are two types of myotonic dystrophy, type 1 and type 2, both of which result from expanded repeat mutations leading to similar molecular pathogenic pathways. The genetic mutation results in expansion containing DMPK RNA which accumulate and interfere with essential cellular processes. The accumulating RNAs appear in the form of foci in the nucleus of the cell, when visualised with in situ hybridization using a fluorescently labelled probe. This study sets out to test and examine the effects of macrolide rapamycin, CDK12 inhibitors and protein phosphatases on quantity of foci and understand the molecular pathogenies of the disorder. In order to test the compounds/therapeutics, immunofluorescence, western blotting, PCR and genome editing using CRISPR Cas9 were some of the techniques used on the patient generated immortalized human KB-TeloMyoD (DM1) fibroblasts, KAGO-TeloMyoD (DM2) fibroblasts. The presence of foci in the DM fibroblasts and the apparent absence of foci in the control/unaffected cells were a definitive indication of genetic mutation and pathology of the disorder. Rapamycin, a macrolide compound was tested in both type 1 and type 2 mutant fibroblasts to determine its effect on the presence of foci. A significant reduction in the quantity of foci was observed in both type 1 and type 2 mutant cells. Further, CDK12 inhibitors, Dinaciclib and 8081 considered as potential therapeutics were tested on HSALR mouse disease models. Dinaciclib produced a slight reduction in foci clusters, however, 8081 didn't show any reduction. Lastly, genome editing using CRISPR Cas9 targeting the catalytic subunits of previously studied protein phosphatase PP1 was conducted to examine its outcome on the foci and DNA mutation. It was observed that even though we observed no evidence of genome editing in any of the catalytic subunits, a slight decrease in the foci was observed in case of PP1CA and PP1CB.

Keywords: Myotonic dystrophy, Foci, Rapamycin, CRISPR Cas9, CDK12, HSALR mouse, protein phosphatases.

1 INTRODUCTION

1.1 Overview

Myotonic dystrophy is a type of muscular dystrophy. It is known as the clinically variable, neuromuscular, multi-systemic, RNA-based, autosomal dominant genetic disorder which affects around 1 in 8000 people worldwide (1-3). Myotonia is a characteristic feature which involves progressive muscle weakness and prolonged muscle contractions. For instance, a myotonic dystrophy patient struggles to relax certain muscles like those involved in releasing the grip on a door knob or a handshake. Skeletal muscles, cardiac muscles, central nervous system and various other body tissues are severely affected in this disorder, thereby resulting in multiple symptoms. Muscle atrophy (shrinkage), respiratory distress, cardiomyopathy and conduction defects (heart failures and abnormalities of electrical signals), arrhythmia, cataract, slurred speech, diabetes, frontal balding, mental retardation are some of the prominent features of the disease (4).

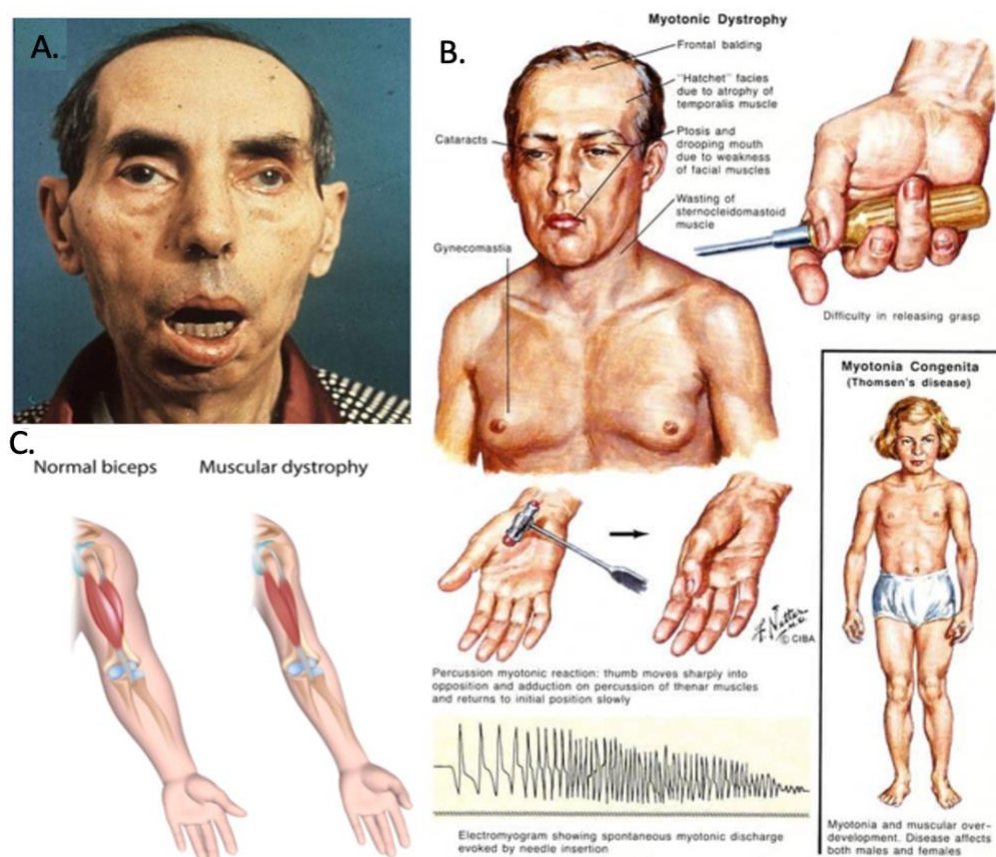


Figure 1.1 : Symptoms of Myotonic Dystrophy. 1.1 (A) Facial features of a myotonic dystrophy patient. Frontal balding and haggard appearance. 1.1 (B) Symptoms including failure in releasing the grip, heart failures and abnormalities of electrical signals. 1.1 (C) Muscle shrinkage in myotonic dystrophy. [Image taken from reference (5, 6)].

1.2 Classification of Myotonic dystrophy

1.2.1 Genetics

Clinically and molecularly, myotonic dystrophy can be classified into two types; myotonic dystrophy type 1 and type 2. Both forms of the disease are inherited in an autosomal dominant pattern and are caused by mutations of expanded nucleotide repeat sequences. However, despite sharing the same root cause behind the disorder, they are genetically quite distinct.

Myotonic dystrophy type 1 (DM1) is associated with the expansion of a CTG triplet located in the 3' untranslated region of the DMPK (DM Protein kinase) gene at chromosome 19q13. Whereas, myotonic dystrophy type 2 (DM2) is caused by a tetranucleotide repeat CCTG located in the first intron of CNBP gene on chromosome 3q21, also known as zinc Finger 9 (ZNF9) (7, 8).

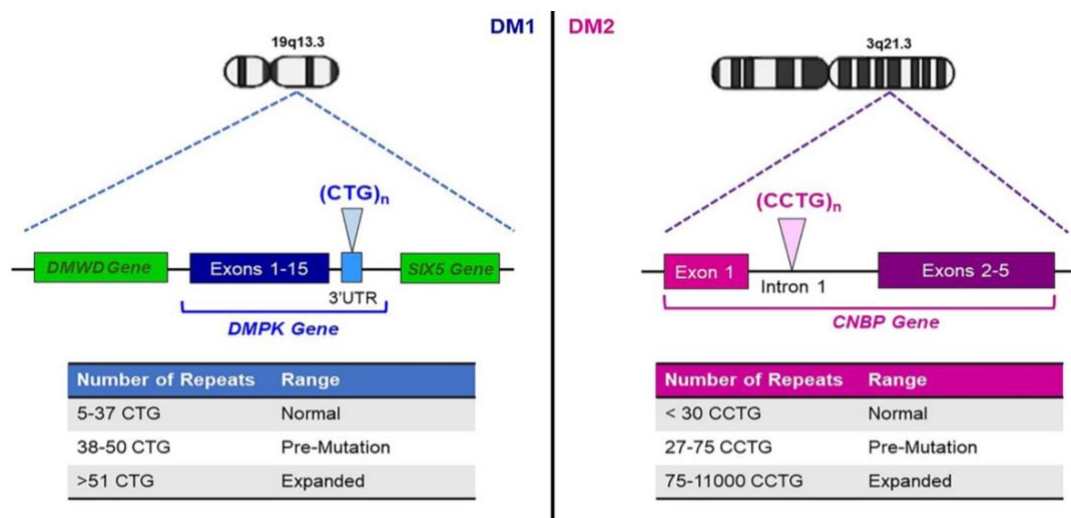


Figure 1.2 : Genetics of myotonic dystrophy type 1 and type 2.

[Image taken from reference (9)].

1.2.2 Disease Severity

Cells of a normal healthy individual usually contains around 5 to 37 repeats in the DMPK alleles or up to 26 repeats in case of CNBP, however, a myotonic dystrophy patient could have repeats ranging up to several 1000 in number (8). Both the disorders

are quite variable with regards to phenotypic repercussions and affect different tissues and systems with varying severity (7). Myotonic dystrophy type 1 predominantly affects distal muscles, that is muscles farther from the center of body such as lower legs, hands, feet and neck whereas muscle weakness in myotonic dystrophy type 2 is associated with muscles close to the center of the body (proximal) like shoulders, hips and elbows (10). Myotonic dystrophy type 1 possesses two main characteristics : (I) Highly instable repeat expansion that persistently generates new alleles with varying repeat sizes. (II) Intergenerational transmission or genetic anticipation, a phenomena where repeat expansion increases in the successive offspring generations (7). Moreover, CTG expansion of DM1 is also highly unstable in somatic cells which accounts for clinical variability. In contrast, DM2 does not possess such a robust intergenerational transmission of repeats. The correlation between age of onset and repeat expansion is also relatively weak, which is why DM2 is almost always milder than DM1 (7, 8, 11).

1.3 Myotonic Dystrophy Type 1

The spectrum of myotonic dystrophy type 1 ranges from fatal effects during infancy to mild symptoms occurring in late-onset period. Considering the complex clinical heterogeneity associated with disorder, the DM1 can be divided into 4 main categories:

- | | |
|-----------------------------------|--------------------------------------|
| (i) Congenital (At birth) | (ii) Childhood-onset (Juvenile) |
| (iii) Classical DM1 (Adult-onset) | (iv) Late-onset/asymptomatic (7, 12) |

1.3.1 Congenital DM1

Congenital DM1 occurs at birth and is the most severe form of myotonic dystrophy type 1 (13). It involves a distinct clinical phenotype. The congenital form is caused by repeat expansion ranging from as small as 750 to expansions with more than 1000 (7). The prenatal symptoms of the disorder are visible in the form of reduced fetal movement and polyhydramnios (presence of increased amounts of amniotic fluid around the baby during pregnancy) (7, 14). After birth the affected infants are floppy/suffer from hypotonia (decreased muscle tone). Other symptoms include facial

diplegia (muscle weakness affecting both sides of the face), clubfeet, feeding difficulties, respiratory support, low visual clarity and more. Intellectual disability, delayed motor and cognitive development are common, which creates the requirement for special schooling (7, 12, 14, 15). Children that survive the early childhood develop symptoms of adult-onset DM1 and go through significant morbidity and mortality while they reach third/fourth decade of their life due to the cardiorespiratory abnormalities (12).

1.3.2 Childhood/Juvenile DM1

Childhood onset DM1 occurs before the age of 12 and the diagnosis is often overlooked due to negative family history and lacking neurological issues (15). Unlike the congenital form where maternal transmission of the disease is more common, childhood DM1 is not influenced by the gender of the parent of origin (12). The characteristics of childhood onset involves facial weakness, however the appearance of tented upper lip is absent. Affected children often suffer from retarded mental development and social negligence leading to variety of psychiatric symptoms including anxiety and mood swings. In addition, cardiac abnormalities like arrhythmias are also evidently observed in this period (7, 12, 14, 15).

1.3.3 Classical/Adult-onset DM1

Adult-onset DM1 is most common form of myotonic dystrophy which produces the symptoms in third/fourth decade of most patients (7). Muscle weakness in classical DM1 is restricted to distal areas and may contribute to symptoms such as myotonia, diabetes, cataract, respiratory failure, cardiac issues, irritable bowel syndrome and more (7, 12, 14, 15). Facial muscle weakness is common thereby producing a typical hatchet appearance with droopy eyes. Pilomatrixoma (skin tumor of hair follicle) and epitheliomas (abnormal growth of epithelium layer of tissue) may be involved (13). Male patients undergo early frontal balding and sometimes may even suffer infertility due to underdeveloped testes (13). Female patients suffer from pregnancy complications including miscarriage, prolonged labor and retained placenta (13).

Various cardiac abnormalities are linked with DM1 which is often the cause for sudden deaths and short lifespan of the patients. Due to multi-symptomatic involvement, patients often suffer from psychological impacts, making anxiety and depression a common phenomenon, and a cause for severe impact on the quality of life (7, 12, 14, 15).

1.3.4 Late-onset DM1

Late-onset DM1 occurs mostly after the age of 40 with repeat size usually between 70 to 100 (7). Most of the patients with late-onset are asymptomatic and often remain undiagnosed until other family member is identified as affected (13). Cataract and balding (men) are the most prominent feature of the disorder however are often misinterpreted due to aging (12). Weakness and myotonia is usually mild. Patients with late-onset have usually normal lifespan but pass on the expansion to their successive generations (7, 12, 14, 15).

Phenotype	Most prominent clinical symptoms	Repeat range	Age of Onset	Life Span	Penetrance and Anticipation (Yes/No)
Pre-mutation	None	38-49	-	Normal	At increased risk of penetrance Uncertain
Late-onset DM1	Cataracts, hypersomnia, myotonia	100-600	>40 years	Normal	Full Penetrance Yes
Adult DM1	Myotonia, cardiac arrhythmias, hypersomnia, gastrointestinal difficulties, muscle weakness and wasting, cataracts, male hypogonadism, insulin resistance, cognitive challenges, left ventricular dysfunctions	250-750	20-40 years	Shortened	Full Penetrance Yes
Juvenile DM1	Similar symptoms as adult DM1 but more severe	400-800	10-20 years	Shortened	Full Penetrance Yes
Infantile DM1	Similar symptoms as congenital DM1 but less severe	500-1100	1 mo. - 10 years	Shortened	Full Penetrance Yes
Congenital DM1	Developmental defects, hypotonia, respiratory insufficiency, cardiac defects, severe cognitive challenges, facial dysmorphism, dysphagia	750-1400	Birth	At increased risk of infant mortality or shortened	Full Penetrance Yes
DM2	Proximal muscle weakness and wasting, cognitive challenges, cardiac arrhythmias, myalgic pain, hypertension	100-10,000	Adult	Normal	Uncertain

Figure 1.3 : Table summarising the clinical features of myotonic dystrophy type 1 and type 2 on the basis of repeat range, age of onset, life span, penetrance and anticipation. [Image taken from reference (8)].

1.4 Myotonic Dystrophy type 2

Myotonic dystrophy type 2, previously termed as proximal myotonic myopathy usually begins between the second and sixth decade of a patient's life (7). The repeat expansion in case of DM type 2 is much larger in comparison to DM1 repeats. It could range from as low as 100 to as high as 10,000 repeats (8). DM2 is known for impacting the proximal muscles like elbow, neck flexor, hips, upper thighs, limb girdle and shoulders (7). The symptoms are highly variable in patients however common features include muscle weakness, myotonia, cataract, diabetes and cardiac abnormalities (12). Insulin resistance and glucose intolerance is present however is lower than in DM1. Excessive sweating and dysphagia (swallowing problems) are also quite common. Cognitive abnormalities include concentration issues, word finding problems and excessive daytime sleepiness (16). Myotonic dystrophy type 2 patients may have a normal lifespan (8), sudden deaths and mortality rate is less, in comparison to DM1. Congenital form in case of DM type 2 is absent. Repeat size, age of onset and penetrance/genetic anticipation are not prevalent in DM2, making it clinically milder than DM1. Patient management in case of DM2 is less understood due to relatively low frequency and large number of patients remaining undiagnosed (7, 12, 14-16).

1.5 Molecular pathology of DM1 and DM2.

As explained in the above sections, the two types of Myotonic dystrophy are linked with two different loci i.e., DMI is associated with DMPK gene whereas DM2 is linked with CNBP. Even though the mutation is located in two different genomic segments, both types have similar disease features and suggest a common pathogenic mechanism (17, 18).

There are three pathogenic mechanisms that explain the repeat expansion mutation with its clinical outcomes :

- i) DMPK and CNBP Haploinsufficiency
- ii) Alteration of neighboring genes
- iii) RNA induced Toxicity (17)

1.5.1 DMPK and CNBP Haploinsufficiency

Haploinsufficiency is a type of genetic dominance where the total level of gene product produced by a cell is not sufficient enough to regulate the normal phenotype thereby resulting in loss of function (19). In case of myotonic dystrophy, patients carry only one wild type copy of DMPK or CNBP gene as the other copy is a mutant carrying repeats, which further reduces the production of a normal DMPK/CNBP protein (17, 18). Therefore, haploinsufficiency could be considered as the potential pathogenic mechanism

to prove the hypothesis of DM multisystem phenotype. However, as stated by (Jansen et al.,1996; Reddy et al.,1996;Berul et al.,1999) in their published paper, the DMPK knock out mice failed to justify the haploinsufficiency as the primer mechanism, as it could not mimic the multisystem involvement observed in the disorder (17). Additionally, CNBP knock out mice also could not reach a reliable confirmed conclusion due to contradictory results. It was then confirmed that haploinsufficiency may be a fair contributor in disease phenotype, but is not the prime reason for it (17, 18).

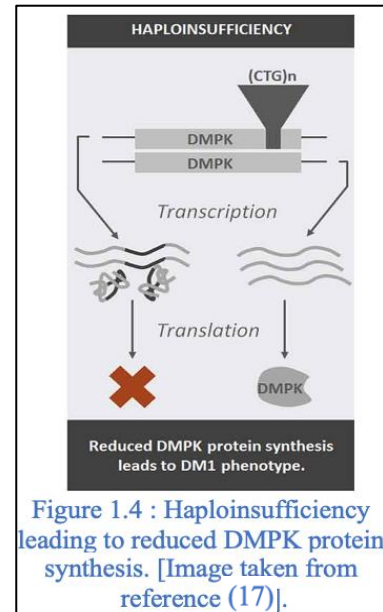


Figure 1.4 : Haploinsufficiency leading to reduced DMPK protein synthesis. [Image taken from reference (17)].

1.5.2 Alteration of neighboring genes

Another proposed pathogenic mechanism behind the disease specific manifestation could be the alteration in the expression of neighboring genes. Tandem repeats are known to interfere with the chromatin structure, and considering the disorder is associated with a mutational repeat expansion it would affect the transcriptional activity of the DMPK and CNBP neighboring genes (17).

In case of DM1 patients, the expression of flanking genes SIX5 and DMWD gets reduced due to the presence of Repeats. In order to confirm the contribution of neighboring genes in the disease expression, a SIX5 knock-out mouse model was created and observed (17).

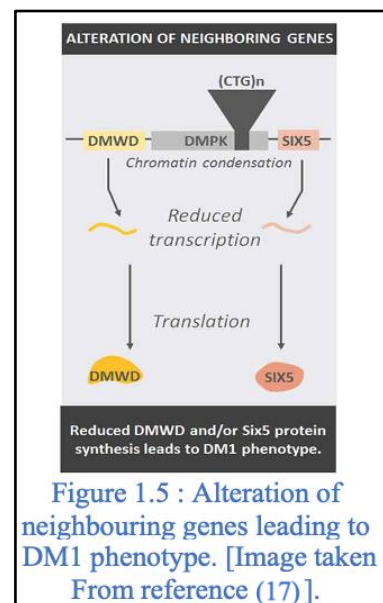


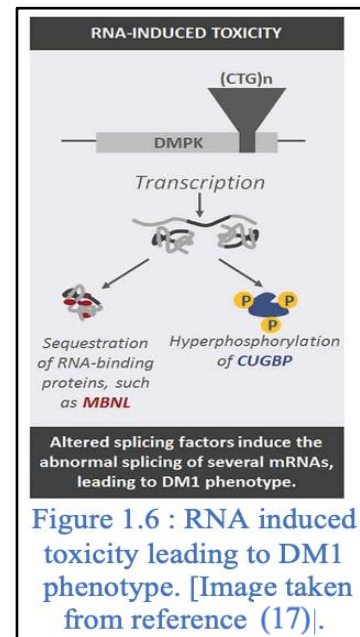
Figure 1.5 : Alteration of neighbouring genes leading to DM1 phenotype. [Image taken From reference (17)].

However, apart from showing an increased risk of cataract, affected cardiac function and reduced fertility in males, the knockout model failed to produce a multi-system involvement characteristic of DM1 (17). Therefore, it was again concluded that changes in the neighboring genes of the DMPK and CNBP is a fair contributor in producing the disease phenotype however, is not the primary mechanism to initiate the disease (17, 18).

1.5.3 RNA induced Toxicity

The third proposed mechanism to explain the molecular pathology of the disease is the RNA induced toxicity (17). Myotonic dystrophy is a cause of a Repeat associated mutation in the DMPK and CNBP genes which after transcription produces the long mutant RNA repeat which is further responsible for the gain of function toxic effects in the cells (17, 18). The expanded CUG/CCUG containing transcripts thereby form a hairpin loop-like structure and accumulate in the nucleus of the cell (17, 18). It then initiates the deregulation of some essential proteins like muscle blind-like protein 1 (MBNL), a RNA binding protein that participate in pre-mRNA transcription and

maturation and gets sequestered to the mutant RNA loop (17, 18). Additionally, the mutant also alters the function of protein CUGBP/Elav-like family member1 (CELF) (17, 18). The hairpin RNA with the sequestered proteins therefore accumulate and clump, and appear in the form of foci in the nucleus of the cell when visualised with in situ hybridization using a fluorescently labelled probe (17, 18). The affected proteins are responsible for the splicing event of various genes, whose altered function results in the production of multisystem involvement characteristic in DM patients. The affected genes include cardiac troponin (cTNT), chloride channel 1 (CLCN1), DNA repair factor MRG15 and more (17). Several mouse models suggest the RNA induced toxicity and foci formation as the main contributing mechanism to the DM multisystem involvement. For instance, the HSA^{LR} (long repeat length of 250) mouse models, that are associated with CTG repeats in the human skeletal actin gene clearly elucidate how foci production play a key role in pathogenesis by establishing the DM phenotype (17).



Thus, It can be concluded that all the three mechanisms participate to some level in producing the DM specific multi degeneration phenotype, however, it is primarily foci production/RNA induced toxic effect that contributes the most (17, 18).

1.6 Potential Therapeutics

There is currently no treatment available to cure myotonic dystrophy, however, the active management of patients is conducted through constant monitoring of the expected complications characteristic of the disease and symptomatic treatments to improve quality of life (15, 20). The understanding of the pathogenesis of the disease has grown over the past years and has concluded that the pathogenesis of the disorder is more complex than known. It thus seems reasonable to target multiple pathways and enzymes with the aim to develop a strategy beneficial enough to combat the DM (20, 21). Hence, the establishment of therapeutics to treat DM1 and DM2 requires the consideration of more in depth knowledge of the molecular pathogenesis and clinical parameters behind the disorder (20).

DM1 is more prevalent and known, and on the other hand, DM2 still remains largely underdiagnosed due to the lack of awareness among clinicians and age related symptoms behind the disease. Even though they produce different genotypical and phenotypical effects, they share a lot similar pathological pathway. Therefore, therapies being established may and may not prove to be effective for both, but will provide a right track towards understanding the pathogenesis of both types of disease and development of effective therapies (18, 22).

The action of molecular therapies could aim at several pathogenic levels of DM :

(1) Potential drugs that could be used to inhibit the production of CTG repeat transcription. (2) Therapies targeting the degradation of mutant RNA to inactivate its further toxic actions. (3) Inactivating / Disrupting the formation of MBNL and mutant repeat complex with CELF regulation. (4) Targeting the affected signaling cascades or pathways like modulation of splicing events and various other pathogenetic processes (22).

Currently, therapies from small molecular compound approaches to Genome editing are being tested to open the door for disease-specific treatments for DM, however, there are still some prevailing limitations of these therapies (11).

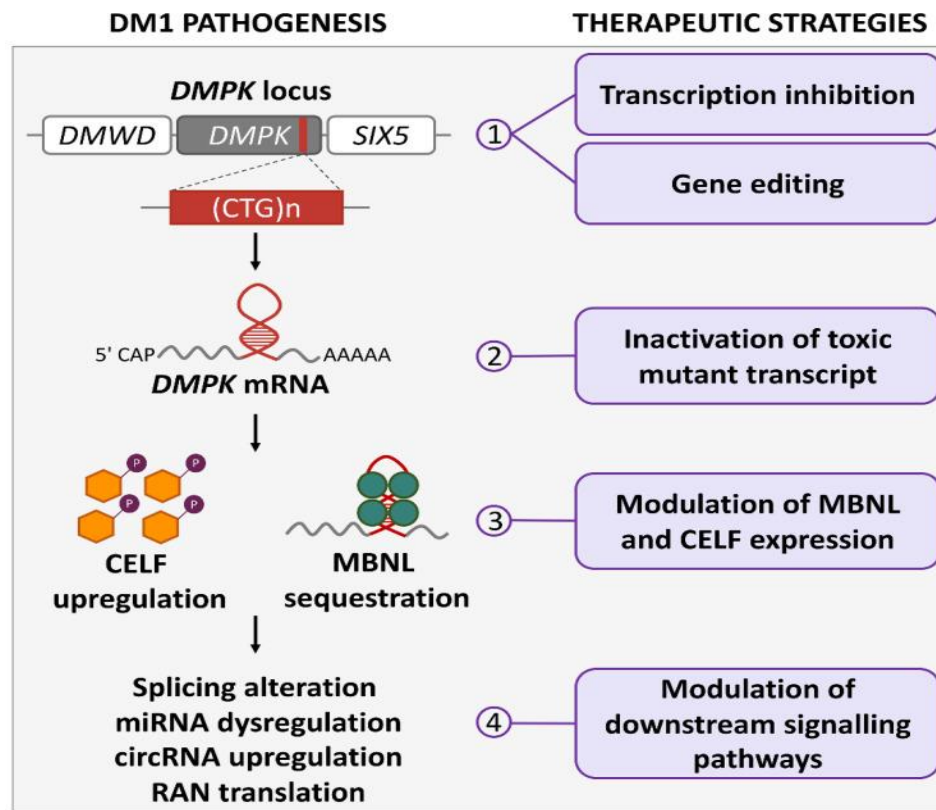


Figure 1.7 : The potential therapeutic strategies of myotonic dystrophy. (1) Inhibition of CTG repeat transcription. (2) degradation or inactivation of toxic mutant transcript. (3) Disruption of MBNL sequestration with the toxic RNA and regulation of CELF. (4) modulation of Downstream Signalling pathways (22). [Image taken from reference (22)].

1.6.1 Small molecular compounds

Due to the cost effective and robust nature of small molecule compounds, they are considered as a preferred pharmacological strategy against DM. Moreover, they can be used to target various pathogenetic mechanisms involved in DM such as degradation of toxic mutant, inactivating MBNL sequestration, modulation of downstream signalling pathways and more (22).

Transcription inhibition and Toxic RNA degradation

Various approaches are capable to modifying the instability of repeats by directly interacting with the repeat mutation and its repair mechanism. Anti-cancer drugs, UV

light, alkylating and intercalating drugs, are some of the DNA damaging agents that are efficient in degrading or suppressing the CTG repeats to induce its deletion (22). Similarly, drugs like caffeine and DNA replication inhibitors hold the capacity to increase mutant repeats (22). Pentamidine, an FDA-approved drug known to treat Pneumonia, sleeping sickness and Leishmaniasis was found useful in disrupting the CUG RNA foci and MBNL sequestration. Pentamidine is a drug that maybe useful in blocking DNA replication by binding to its minor groove, however, the hypothesis behind the foci reduction was attributed to decrease in the transcription of CUG transcripts or the upregulated destruction of toxic RNA (4, 21). Actinomycin D, an anti-cancer FDA approved polypeptide antibiotic is known for inhibiting global transcription via binding to DNA duplexes (4, 21, 23). Moreover, diluted doses of Actinomycin improved the DM1 molecular phenotype (4, 21). The produced effects imply the obstruction of RNA polymerase activity and reduced transcription expression due to its known characteristic of intercalating GC rich sequences thereby impeding the RNA polymerase progression (21). Mexiletine, prilocaine and procainamide also inhibit the DMPK transcription (4). Further, Triaminotriazine compounds such as ligand 1, ligand 3, and ligand 9 also target the repeat expansion and produce results by the capability of transcription inhibition and cleavage of CUG RNA thereby reducing the ribonuclear foci (4, 23). Microtubule inhibitor colchicine is also reported helpful in selective modulation of toxic CUG RNA by transcriptional inhibition (22).

MBNL and CELF modulation

Small compounds inhibiting MBNL sequestration with repeats, or modulating its expression disperses RNA foci and DM phenotype (21). Nonsteroidal anti-inflammatory drugs like Phenylbutazone (PBZ) and Ketoprofen used for pain, fever, and blood clots, Chloroquine anti-autophagic drug and recombinant-adeno-associated virus (rAAV) vectors carrying MBNL1 are some of the small molecular compounds that have the potential to reverse the myotonia, repair the aberrant splicing and downregulate the DM phenotype by alleviating the expressions of MBNL1/2 proteins in the cells (21, 22). Some medications like histone-deacetylase inhibitors induce activation of MBNL1 expression and thereby help in DM treatment (22). The HDAC inhibitors ISOX and Vorinostat also significantly increase the MBNL protein levels and help in partial rescue of the disease specific phenotype (4, 21, 22). Furthermore, targeting CELF1 regulation indicated improvements in DM. PKC inhibitors and CELF

upregulation could potentially be used to rescue CELF dependent splicing defects, contractile dysfunction and mortality (22). The DM pathogenesis induces deregulated CELF1 levels and a dysregulated balance between Glycogen synthase kinase 3 β (GSK3 β) and its negative regulator cyclin D3. Hence, GSK3 β inhibitors : lithium and 4-benzyl-2-methyl-1,2,4-thiadiazolidine-3,5-dione (TDZD-8) can reduce the myotonia by restoring a balance between the levels of cyclin D3 and CELF. Additionally, an oral GSK3 β inhibitor medication Tideglusib (TG) also produced positive significant results in reducing DM phenotype (4, 21, 22).

Downstream signalling cascades

Reports suggest that treatments targeting downstream signalling cascades can be helpful for DM therapy. Since, insulin resistance and predisposition of type 2 diabetes is a symptom of both DM1 and DM2, therapy targeting Insulin rector IR were tested (20). Anti-diabetic drug metformin was shown to correct abnormal IR splicing in DM1 mesodermal precursor cells and in DM1 myoblasts. Metformin was also used to treat DM1 patients in a phase II clinical trial. However, drug's failed attempt to improve myotonia and its side effects raised question to whether metformin helped in DM1 treatment (4, 20). Further, drugs targeting imbalanced mTOR/AMPK in DM were tested. AICAR, activator of AMPK and Rapamycin, an mTOR inhibitor produced a reduction in foci and improved muscle function or myotonia in HSA^{LR} mouse. Age-associated drug Resveratrol (RSV) which activates AMPK also produced significant positive effect on splicing in HAS^{LR} mice (4, 20, 22). Erythromycin, a macrolide compound reduced myotonia and other symptoms of DM1 and is being tested in clinal trail phase II. The drug is known to restore splicing defects and MBNL activities in the mouse models (4). Drugs targeting repeat associated non-ATG (RAN) translation also represented a promising target against DM. 1H-5-CA-biotin was tested and improved pre-mRNA splicing defects and inhibited RAN translation in a neurodegenerative disorder caused by repeat expansion. Therefore, drug targeting RAN translation is an attractive venture for researchers (22, 23).

1.6.2 Antisense Oligonucleotide ASO's and Small Interfering RNAs (siRNAs)

ASO's and siRNAs are the two most widely used gene silencing strategies that share a fundamental principle. Antisense Oligonucleotide ASO's are an antisense therapy that use the short synthetic nucleic acids to target and hybridize with messenger RNA via Watson-crick base pairing to modulate the expression of genes (24-26). It follows a sequence specific binding leading to an altered protein production by attacking the disease source at the RNA level. Similarly, Short interfering RNAs (siRNA) are artificially synthesized RNA molecules that target the transcript via sequence complementarity and modulate the expression of genes by eliciting a RNA interference (RNAi) response (24-26). Distinguishingly, ASO's are delivered as single stranded oligonucleotides that function to identify and hybridise with the target independently whereas siRNAs on the other hand are delivered in duplex which then with the help of Argonaut AGO forms a RNA silencing complex, guiding the leading strand towards the target and leaving the passenger strand behind (24-26).

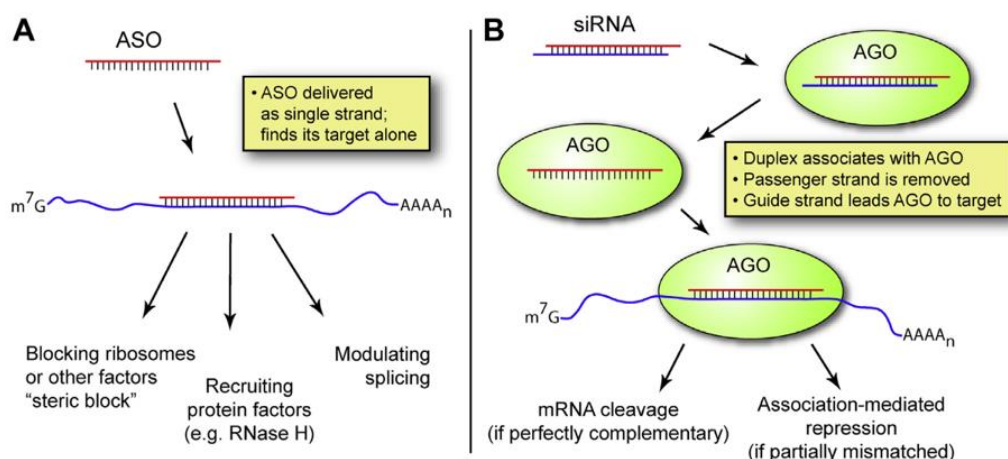


Figure 1.8 : Comparison of the ASO and siRNA mechanisms (24). [Image taken from (24)].

Since, the unmodified ASO's and siRNAs are susceptible to degradation via nucleases in blood serum and elimination through renal filtration, chemical alteration are incorporated into the ASO's which not only enhances their stability and efficacy in in-vivo applications but also enables its direct administration thereby reducing manufacturing expenses (22).

Gene silencing via ASO's and siRNAs in myotonic dystrophy is targeted with mainly two aims: (i) To block MBNL-CUG interaction (ii) Degradation of mutant expansion. The removal of the mutant RNA using ASO's and siRNAs is considered a logical strategy to combat the disorder as the degradation of the mutant is expected to repair the following toxic downstream signalling cascades and consequently help in rescue of the disease pathology (21). ASO's can produce a steric hindrance in the gene expression by promoting RNase H-mediated cleavage or degradation. Ribonuclease H (RNase H) is a non-sequence specific enzyme which targets the RNA-DNA duplexes, however only catalyses the degradation of the RNA strand (27, 28). Since, myotonic dystrophy pathophysiology has a major correlation with the production and accumulation of defective RNA, availing an ASO complementary to a specific RNA sequence could help in the degradation of the target RNA, and eventually in rescuing the disease. Chemical modifications involving locked nucleic acid (LNA), 2'-O-methoxyethyl RNA (2'-MOE) and 2',4'-constrained 2'-O-ethyl bridged nucleic acid (cEt) placed at both ends of ASO also helps in the increased affinity and degradation of the target RNA (22, 27). Several reports conveyed that modified ASO's were successful in producing results favouring the rescue of the disease and symptoms. Morpholino ASO binds to toxic CUG expansion and blocks MBNL sequestration, 2'-MOE modified ASO's were shown to deplete the levels of toxic RNA, gapmer associated ASO's with CUG repeats produced significant results in both cells and mouse models. DMPK-specific ASO injected into DMSXL mice through intracerebroventricular injections proved success through reduced levels of toxic RNA and favourable alteration of behavioural abnormalities (22, 27).

Similarly, siRNA injections induced in HSA^{LR} mice produced significant reversal of DM phenotype, with decreasing levels of transcript via the RNA interference pathway which is capable of depleting the protein translation through RNA specific binding. Engineered hu7-siRNAs carrying poly CAG antisense sequence were successful in degeneration the toxic expansion in DM cells. Further, siRNA conjugated with transferrin receptor 1 (AOC 1001) aiming the reduction of DMPK repeats is being tested in clinical trials due to its efficient uptake in skeletal and cardiac muscles (22).

After several methods and modifications, the applications of gene silencing proved to be successful in preclinical studies, by reducing the mutant RNA, decreasing myotonia

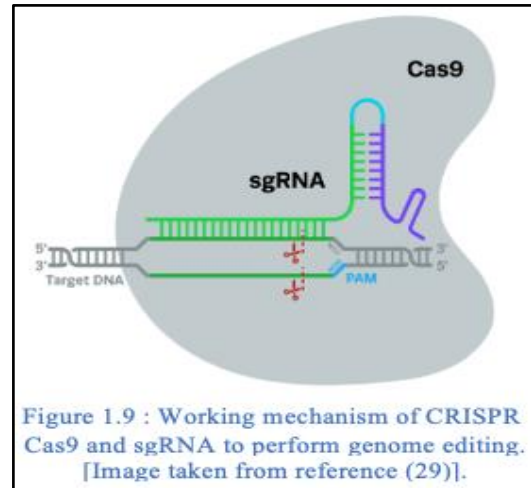
and improving DM associated events. However, despite the successful testing, ASO's and siRNAs still demand a better and effective conveyance to the skeletal muscles, attracting a need for new and improved approach. Delivery approaches involving intracerebroventricular injections and conjugate antibodies are being tested. Additionally, dosage tolerability, safety, reflex immune response, toxicity, and target specificity are yet the prevailing and essential problems to be accomplished before they can successfully be used as therapeutics against myotonic dystrophy (11, 20, 22, 27, 28).

1.6.3 Genome Editing Therapy

The most ideal and logical way to cure myotonic dystrophy is to directly eliminate the CTG/CCTG repeat from the DNA itself. This approach highlights the benefit of potentially eradicating the DM mutation from the genome thus averting any further downstream toxic effects, however, also remains the most challenging endeavour (20).

Engineered DNA-cleaving enzymes such as zinc-finger nucleases (ZFNs) and transcription-activator-like effector nucleases (TALENs) are some of the potential genome editing therapeutics that could be used. Double stranded breaks in CTG repeats induced by ZFNs were shown to produce significant deletions in yeast, however, increased and duplicated the repeats in case of mammalian cells indicating the need to target the repeat flanking regions. Similarly, double stranded breaks produced by TALEN also proved successful in repeat contractions in yeast. TALEN system are also being utilized to induce a polyadenylation (polyA) signal upstream of CTG repeats which result in early cessation of transcription and help in getting rid of toxic mutant transcripts. However, unfortunately, reports conveyed that the toxic CTG repeats retained at the genomic level due to TALEN target-sequence constraint, creating a need for further research (22). On the other hand, CRISPR (clustered regularly interspaced short palindromic repeats) with the help of Cas9 provide a more simpler and flexible approach towards editing the genome. CRISPR exploits the cleaving capabilities of the *Streptococcus pyogenes* CAS9 (SpCas9) or the *Staphylococcus aureus* cas9 (SaCas9) endonucleases by targeting them towards a specific DNA sequence and produce a double stranded break or alteration in the gene (20, 22). The cas9 protein is guided by

their respective single-guide RNAs (sgRNAs) which is short RNA sequence complementary to the target sequence. Together, they form a ribonucleoprotein (RNP) complex that helps in cleaving a specific target. The resultant break is then allowed to be repaired, finally resulting in gene knockout or knock in (20, 22, 29).



Various genomic editing techniques are being tested in case of myotonic dystrophy and the main strategies proposed to curb the toxicity of DM involve :

- (i) Transcription termination using deactivated Cas9 (dCas9) with sgRNA aiming the CTG repeats
- (ii) Single stranded CUG RNA degeneration using RNA-targeting cas9 (RCas9)
- (iii) Premature polyA signal insertion in the DMPK gene (20, 22).

A successful repression of nuclear foci, mutant RNA transcript, splicing defects and DM toxic events were achieved in all the strategies mentioned tested on DM1 cells. Rcas9 and inactive CAS9 also produced significant reduction of DM phenotype in HSA^{LR} mouse models (20, 22). It was also reported that recombinant adenoviral vectors carrying Cas9 nuclease along with sgRNA, injected intramuscularly in DMSXL mice successfully eliminated the CTG repeats in the muscle. Additionally, CRISPR Cas9 was able to produce rewarding results in many cell models including patient derived immortalised DM myoblasts, immortalised myoblasts generated from DM1 transgenic animals and MYOD1-convertible fibroblasts. Thus, genome editing highlights its great potential towards producing a reliable therapy against myotonic dystrophy (20, 22).

Unfortunately, various limitations still prevail in utilizing genome editing techniques such as off target effects, its impact on function of neighbouring genes, specificity, and safe and efficient delivery. Moreover, a multisystemic disorder would require the delivery of CIRSPR Cas9 in all the affected tissues. Therefore, even though genome editing hold a potential for curing myotonic dystrophy, additional work and focus would be required to eliminate all the limitations (11, 20, 22).

1.7 Rapamycin and mTOR pathway

1.7.1 Rapamycin Drug

Rapamycin clinically known as sirolimus or rapamune belongs to a macrolide class of antibiotics produced by *Streptomyces hygroscopicus* (a bacterial species isolated from the soil samples of Easter island by George Nogrady in 1960) (30-32). Rapamycin is known for its antifungal, anticancer and immunosuppressive properties. The drug possess the characteristic ability to subdue the immune system leading to its usage for the prevention of rejection during renal organ transplantation. FDA approved the drug in

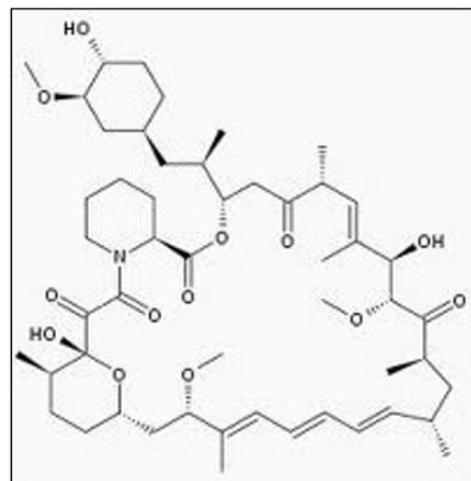


Figure 1.10 : Chemical structure of Rapamycin.[Image taken from reference (32)].

1994 for its antirejection properties, followed by an FDA approval for the drug's utilization in cancer treatment such as Lymphangioleiomyomatosis (30, 31, 33-35). Further, rapamycin is a specific inhibitor of mTOR (mammalian target of rapamycin). It forms a complex with FK-binding protein 12 (FKBP12) also called as an immunophilin and binds and inhibits the activity of mTOR which plays a fundamental role in the cell cycle (31). Rapamycin is thus able to disrupt the cell division by deactivating the mTOR protein. Rapamycin also holds the potential of inhibiting angiogenesis which has been successfully proved by extending lifespan and weakening aging and disease progression in multiple organs of various model organisms (30, 31, 33-35).

1.7.2 mTOR Pathway

Mammalian target of rapamycin mTOR is a highly conserved serine-threonine kinase, and is considered as the master-regulator governing the response of eukaryotic cells to stimuli such as nutrients, cellular energy, growth factors and stress. It engages in various signaling events in the body to regulate cell proliferation, autophagy and apoptosis (36, 37). mTOR comprises of two structurally and functionally distinct multi-

protein complexes : mTOR complex I (rapamycin sensitive) and mTOR complex II (rapamycin insensitive). mTOR I includes mTOR, raptor, GβL and deptor and is responsible for cell metabolism and energy homeostasis. mTOR, when activated, converts the cell metabolism from catabolic to anabolic resulting in the production of protein, lipids and nucleotides with a repressing level of autophagy. On the other hand, mTORC2 is composed of mTOR, Rictor, GβL, PRR5, deptor, and SIN1 which majorly functions to manage cell proliferation and survival (36). The mTOR orchestrates transcription and protein synthesis by responding to various stimuli and regulating cellular processes, hence, any unbalanced regulation in the process leads to consequent disorders. Various studies indicate the involvement of mTOR signalling pathway with a range of diseases including tumours, arthritis, osteoporosis, and more (36, 37).

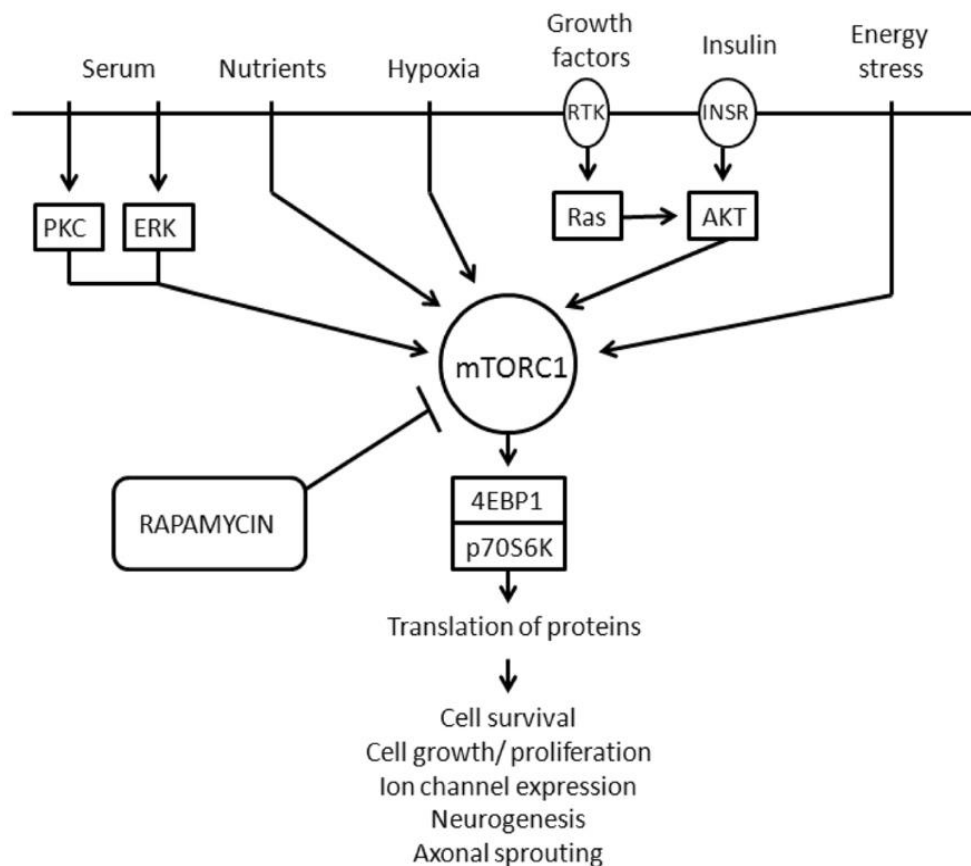


Figure 1.11 : **A simplified version of the mTOR signalling pathway.** MTORC1 receives and integrates the signals from various upstream pathways, including pathways triggered by nutrients, growth factors, hypoxia and insulin that promote cell growth, division and differentiation. Rapamycin is a powerful inhibitor of mTORC1 activity. Abbreviations: 4E-binding protein (4EBP1); eukaryotic translation initiation factor 4E (eIF4E); Receptor Tyrosine Kinase (RTK); Insulin receptor (INSR); Protein Kinase C (PKC); Extracellular Signal Regulated Kinase C (ERK); (Ras); Protein kinase B (AKT) (37). [Image taken from reference (37)].

1.7.3 Rapamycin's mode of action in myotonic dystrophy

Abnormally enhanced/deregulated mTOR signaling has been reported in several animal models suffering from myopathies including DM1. DM1 has been reported with signs of increased autophagic flux and imbalance in the ubiquitin-proteasome system, both of which link to muscle weakness in the disease. Moreover, the glucose intolerance in DM1 alter the insulin receptor expression levels which affect the PKB/Akt pathway explaining a correlation between the deregulated mTOR and disease phenotype (38). It was later confirmed that HSA^{LR} mouse do not respond to mTOR efficiently and indicate the presence of impaired AMPK activation and delayed inhibition of mTOR complex 1. Further, it is possible that the deregulation observed in autophagic flux is also due to AMPK/mTORC1 imbalance (39, 40).

An improved state of myotonia and skeletal muscle strength was reported when treatments regulating the mTOR pathway were tested. AICAR (AMPK regulator) and rapamycin (mTOR regulator) treatments proved beneficial in restoring the balance in mTOR pathway and highlighted its potential utilization against myotonic dystrophy (39).

1.8 CK12 Involvement in myotonic dystrophy

Cyclin dependent kinase 12 (CDK12) is a protein kinase comprising of 1490 amino acids encoded by the *CDK12* gene. It is a member of the serine/threonine protein kinase group of the CDK family and collaborates with cyclin K to ensure the implementation of various cellular processes. CDK12 is known to be involved and overseeing cell cycle, gene transcription regulation, RNA processing and controlling translation (41, 42).

CDK12 RNA produces two transcripts of 8.5 and 6.8 kb respectively and are expressed in all human tissues. Further, CDK12 protein has proven to be quite essential in both adult and developmental phases (41). Various kinases are involved in the regulation of phosphorylation of the C-terminal domain of RNA polymerase II, during the process of

transcription which includes the contribution of CDK12. It promotes the phosphorylation of Ser2 at the C-terminal domain of RNA polymerase II which helps in maintaining genome stability and thereby facilitating transcription. Therefore, CDK12 plays an important role in the elongation event of transcription (2, 41).

CDK12 is involved in the process of mRNA processing via two ways : (i) Direct association with mRNA processing factors (ii) Recruitment of factors involved in splicing and 3'end processing through CTD phosphorylation. It also modulates the alternative last exon (ALE) splicing process. SRSF1 and c-MYC are some examples of genes whose RNA processing is CDK12 regulated (41).

Hence, inhibitors of CDK12 illustrate the potential as therapeutic targets against various human cancers, myotonic dystrophy1 and other diseases (42). CDK12 inhibitors if used can premature terminate the transcription of gene involved in DNA repair and replication. As CDK12 is not required at the beginning of the transcription, it does not cause a global transcriptional arrest thereby showcasing its possible suitability as a target for long term DM1 treatment. Further, it can also be used to downregulate fully transcribed protein coding mRNAs which could prove to be a potential mechanism to combat various disorders (2).

Ketley et al. successfully demonstrated the link between CDK12 and myotonic dystrophy. The number of nuclear foci in DM cells as well as HSA^{LR} mouse models significantly reduced or eliminated via CDK12 inhibitors which highlights its potential as therapeutic targets (2). Ketley et al. also demonstrated a 48% increase in the level of CDK12 protein in DM1 muscle samples compared to healthy muscle samples. It was further observed and proved that both CDK12 and nuclear foci co-localize with nuclear speckles, reasoning the authors to quantify the CDK12 granular structures in both DM and healthy cells, which showed a similar increase. Therefore, it was proved that CDK12 overexpression in DM1 cells increased the number of nuclear foci (2). Similarly, a significant decrease of 69% and 56% of nuclear foci and CDK12 granules in DM1 cells respectively was observed via knockdown of CDK12 using shRNA. A genetic knockdown of CDK12 could produce a decrease in nuclear foci by either suppressed transcription or increased degradation of mutant transcription (2). However, It was

concluded that CDK12 inhibition primarily effects transcription rather than degradation of the formed transcripts through the attained ddPCR results (2, 41).

The potential results of CDK12 inhibition were further validated by treatment of DM1 cells with CDK12 inhibitor Dinaciclib, SNS-032 and THZ531. Dinaciclib and SNS-032 have been used in clinical trials for cancer therapies, hence validating their potential against Myotonic dystrophy. When tested in DM1 cells, both dinaciclib and SNS-032 produced significant results in reducing nuclear foci against non-treated cells (2, 41). To examine the potential of dinaciclib treatment, it was further tested in HSA^{LR} mouse models. Blind analysis was performed to ensure unbiased results and dinaciclib successfully produced a decrease in the nuclear foci in comparison to non-treated mouse samples (2, 41).

1.9 The Protein Phosphatases as potential therapeutic targets

1.9.1 The diversity of protein phosphatases

The basic foundation of any biological event is the strict and controlled regulation of the activities of thousands of proteins. Protein phosphorylation is an essential reversible and dynamic post-translational modification that carries the potential to alter the fate, localization, folding, stability and the interaction of cellular proteins (38). Protein kinases are responsible for the phosphorylation state of a protein i.e. function to add a phosphate group to a protein to activate or inhibit any cellular event. In contrast, protein phosphatases carry the responsibility to dephosphorylate a protein that is to remove a phosphate group from the protein to transfer it back to its unphosphorylated state and maintain the balance. Any perturbations or imbalance in the regulation of kinases and phosphatases can severely impact the cellular processes including survival, growth, migration, differentiation and energy metabolism thereby associating with several human diseases like cancer, diabetes, Alzheimer and neurological disorders. Various reports suggest that myotonic dystrophy is also one of the disorders to have altered phosphorylation and expression levels (38). Phosphatases are widely expressed in

immune system, and given their key role in the same, the enzyme super family holds potential as drug targets to combat autoimmune diseases (38, 43).

However, phosphatases are promiscuous in nature and misconceptions regarding their specificity and activity has led them to be overlooked by drug discovery programs for years whereas kinases have always been the top targets in pharmaceutical industry. But, just as a cellular process requires regulated kinases, it also needs regulated phosphatases. The kinase mechanisms control the assembly and docking and the phosphatases ensure the accurate timing and prevent any untimely activation of complex signaling pathways (44-46).

The phosphatases are obligate multimeric enzymes that have several catalytic subunits combining with hundreds of regulatory subunits. The combination of both is what leads to precision from promiscuity, making them unambiguously targeted enzymes that coordinate highly complex biological events. The regulatory subunits rather than the catalytic subunits determine the substrate specificity, subcellular localization and fine tuning of phosphatase activities (44-46).

1.9.2 Classification of protein phosphatases

The protein phosphatases can be subdivided on the basis of substrate specificity into families : Serine/threonine specific phosphatases and tyrosine specific phosphatases. The serine/threonine phosphatases are further divided into subfamilies due to their diverse structural and functional nature. The serine/threonine phosphatases include PPP phosphoprotein phosphatases, PPM Mg^{++} dependent protein phosphatase and aspartate based protein phosphatases. On the other hand, protein tyrosine phosphatases are subdivided into groups called: cytosolic non receptor form, transmembrane receptor like form, low molecular weight form and dual-specificity phosphatases (47).

Furthermore, majority of serine/threonine phosphatases are attributed to two members of the PPP family : PP1 protein phosphatase 1 and PP2A protein phosphatase 2A. The tight regulation of specificity and distinct cellular localization in case of PPP family is determined through the association of catalytic subunits with the non-catalytic subunits

forming multimeric holoenzymes. PPPs holoenzymes expand their association with regulatory protein which allow them to be tightly selective (44-46).

Unfortunately, these regulatory subunits have not been studied well which highlights the prevailing major challenge to find ways to better identify and understand the phosphates regulatory circuits in order to use them as potential therapeutics against diseases (44-46).

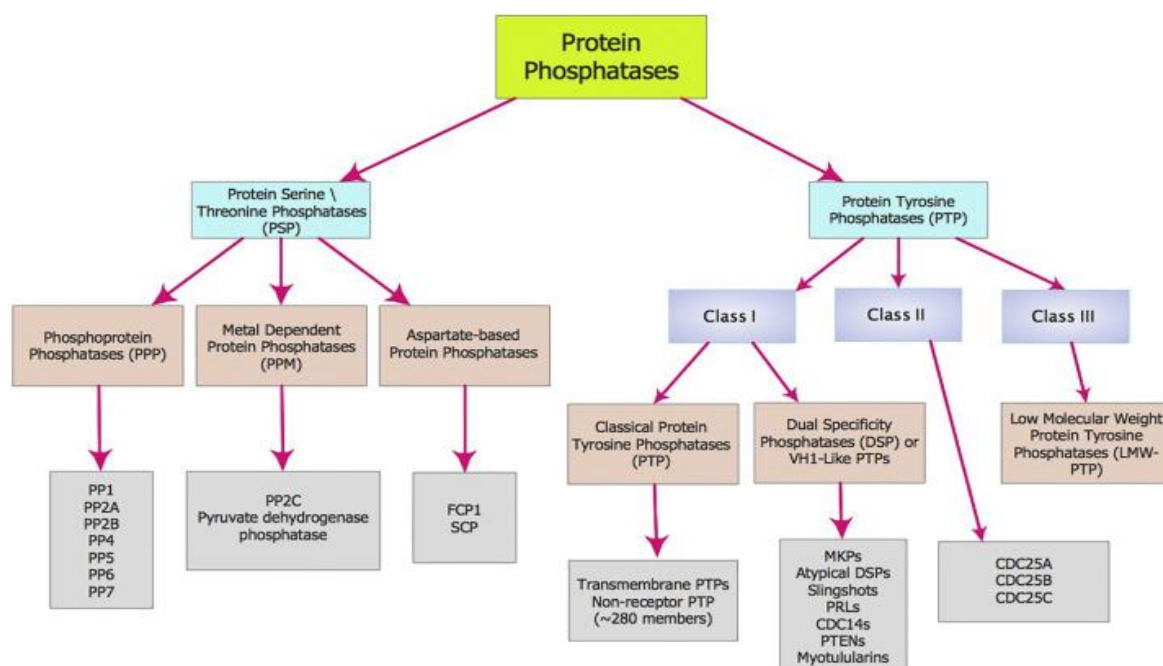
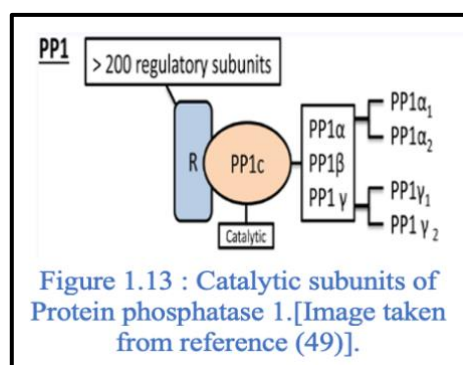


Figure 1.12 : Classification of the protein phosphatases.

Abbreviations: CDC14s, protein tyrosine phosphatase CDC14; FCP-1, TFIIF-associating component of RNA polymerase II CTD phosphatase; MKPs, mitogen-activated protein kinase phosphatases; PRLs, phosphatases of regenerating liver; PTENs, phosphatase and tensin homologs; SCP, small CTD phosphatase (48). [Image taken from reference (48)].

1.9.3 Protein phosphatase 1

There are four PP1 genes in the human genome which encode for the catalytic subunits isoforms : PP1C α , PP1C β , PP1C γ_1 and PP1C γ_2 . PP1C γ_1 and PP1C γ_2 are a product of alternative splicing, which also gives rise to two PP1C α isoforms (49). The four isoforms share >90% sequence identity, however, have different tissue



expression and functions due to its association with different partners. The substrate specificity in PP1 is ensured through the combination of catalytic subunits with diverse set of >200 regulatory proteins (46). Hence, even subtle difference in the associated regulatory subunit can lead the phosphatase to a different substrate or location in the same subcellular surrounding. A regulatory subunit may act as both, a regulator and an inhibitor which explains the distinctive functions of phosphatases (46, 50, 51).

PP1 is a key regulator of several cellular biological processes which includes cell cycle, protein synthesis, pre-mRNA splicing, transcription, apoptosis and more. PP1 also reverses translational control mechanisms, promotes recycles of transcription, and deactivates certain splicing factors. It is therefore evident that PP1 plays a crucial role in human pathologies and holds a great therapeutic potential against various diseases like cancer, heart disease, diabetes and more (50-52).

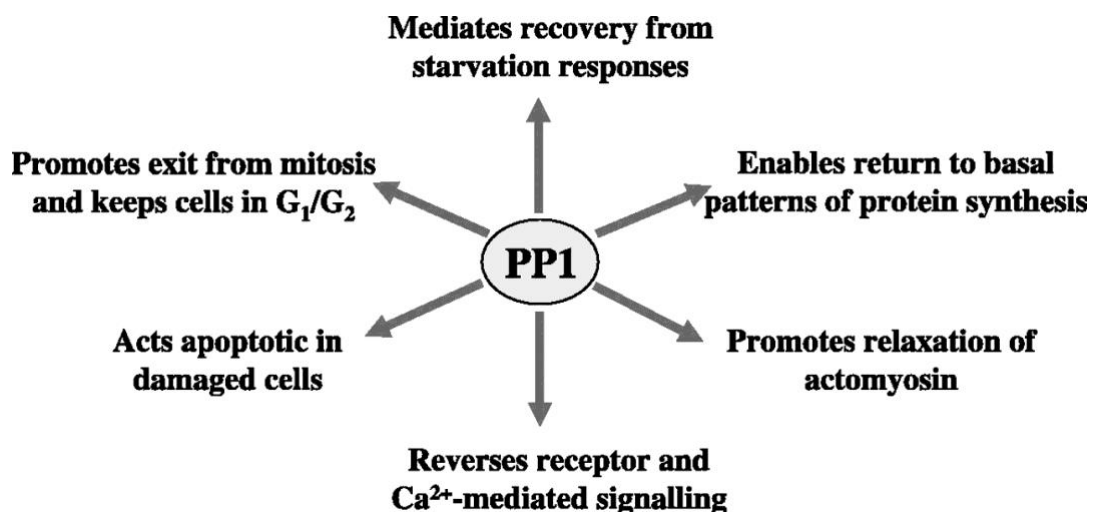


Figure 1.14 : Functions of PP1 (51). [Image taken from reference (51)].

2 AIMS OF THE PROJECT

The study aims to test relevant compounds or therapeutics on patient generated immortalized human KB-TeloMyoD (DM1) fibroblasts and KAGO-TeloMyoD (DM2) fibroblast and wildtype control fibroblast cells to examine the effects on nuclear foci and understand the molecular pathogenies of the disorder.

- **Compound Treatment**

Rapamycin, a macrolide mTOR inhibitor compound is used to test its effect on the presence of foci in both type 1 and type 2 mutant fibroblasts at different concentrations via fluorescence in-situ hybridization.

- **CDK12 Mouse Treatment**

CDK12 inhibitors, dinaciclib and 8081 considered as potential therapeutics were injected into the HSALR mouse disease models. Gastrocnemius muscle tissues from these mice were tested via immunofluorescence to analyse their effects on the number of foci.

- **Genome editing via CRISPR**

Gene silencing via SiRNA and genome editing using CRISPR Cas9 targeting the three catalytic subunits of protein phosphatase PP1 was conducted to examine its outcome on the foci and DNA mutation using fluorescence in-situ hybridisation, western blot and PCR.

3 METHODOLOGY

3.1 Tissue culture

3.1.1 Cell Line

Immortalized human KB-TeloMyoD fibroblasts and KAGO-TeloMyoD fibroblast cells, curated from patients suffering from myotonic dystrophy type 1 and type 2 respectively, were cultured to test the effect of potentially therapeutic compounds on them. Additionally, immortalized human SB fibroblasts cells were also cultured and used as positive control in some experiments.

3.1.2 Cell Culture

The cells were cultured in Dulbecco's modified eagle's medium (DMEM) media (500ml) supplemented with 15% Foetal bovine serum (FBS) (75ml) and 2% of 100X penicillin streptomycin (10 units of Penicillin and 10mg Streptomycin per ml in 0.9% NaCl 0.1µm filtered) (12ml) . T-75 flasks were used to grow the cell lines and were incubated at 37°C with 5% CO₂. Cells were split every once or twice a week, depending on their growth and confluency levels to allow their efficient growth and survival. In order to split the cells, The old media from the flask was removed followed by a 1X phosphate buffer saline (PBS) wash (10ml) . Further, trypsin-EDTA (3ml) was used to separate the cells from the flask. Leaving 0.5ml of trypsin in the flask, with incubation at 37°C, the cells were allowed to trypsinise for 5 mins. The cells were then split and resuspended in the culture media equally among the 2-3 new labelled T-75 flasks containing 20ml media in each.

3.1.3 Freezing the cell line

To freeze the cells, the media from the T-75 flask containing cells was removed, followed by a 10ml PBS wash. The cells were then trypsinised for 5 min at 37°C and resuspended in 10ml DMEM media. The cells were transferred to a sterile universal container, and centrifuged for 5 minutes at 1000g. Next, the supernatant media was

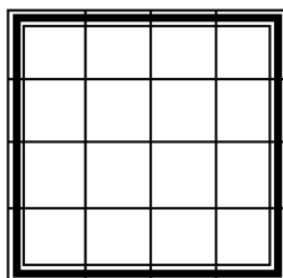
removed carefully, leaving the pelleted cells at the bottom. The pelleted cells were gently suspended in 10% DMSO, and transferred to a labelled screw capped vial. The vial was stored at -80°C for 24 hours after which it was transferred and stored in the liquid nitrogen.

3.1.4 De-freezing the cell line

To defrost the cells from liquid nitrogen, the frozen cells in the screw capped vial were thawed using a water bath set at 37°C temperature. The cells were suspended in 1ml of pre-warmed DMEM culture media and immediately transferred to a sterile universal container containing 10ml of DMEM media. Rapid transfer is essential to avoid any cell death due to DMSO toxicity. The cells were then centrifuged for 1 minute at 1000 rpm. The supernatant media is carefully removed leaving the pelleted cells which are again resuspended in 10ml of new media and transferred to a T-75 flask. The T-75 flask is added with some media (final volume 20ml) and incubated at 37°C for optimal growth.

3.1.5 Cell Count using Hemocytometer

Cells were trypsinised for 5 mins at 37°C with a 10ml PBS wash in between, using the similar protocol as mentioned above. Further, cells were resuspended in 10ml of DMEM media and 0.5/1ml of it was pipetted out. The hemocytometer was then loaded with the cells by placing the tip of the pipette at the edge of the chamber and allowing it to fill. The hemocytometer was then placed on the microscope with 10X objective and was focused to observe the chamber grid lines. The total number of cells occupying square area of 1mm² were counted. This was performed 3 times and an average value of it was taken into account.



1 unit = 1mm²
square

Formula :
No. of cells per ml = No. of
counted squares X 5000

3.2 Compound Treatment Rapamycin for DM1 and DM2 cells

3.2.1 Fluorescence in-situ Hybridization

Cell plating

Both KB/KAGO and SB fibroblast cells were plated in the 96 multi-well Greiner plate (Greiner Item-No 655090). Cells were trypsinised and counted using the hemocytometer as mentioned above. Depending on the confluency of the cells, the volume of cells per well was calculated. In my project, the calculations were guided by the consideration that each well contained around 5000 cells. The plated cells were then left for 48 hours in a moist condition at 37°C in the incubator with 5% CO₂.

Rapamycin Drug Dosing

48 hours post cell plating, the cells were dosed with Rapamycin (stock concentration of 10mM) at various Concentration through ½ fold serial dilution; ranging from highest concentration of 200uM to lowest of 12.5uM. Additionally wells were dosed with DMSO at different concentrations that worked as negative control for the cells. The cells were treated for 24 hours. The plated SB fibroblast cells were kept untouched and acted as positive controls of the experiment. The table below depicts the volume and concentration of rapamycin and DMSO control doses.

RAPAMYCIN TREATMENT (CONCENTRATION)	½ fold Serial dilution ↓	DMSO CONTROL (PERCENTAGE AND VOLUMES)
200 uM		2% DMSO ; 4 ul
100 uM		1% DMSO ; 2 ul
50 uM		0.5% DMSO ; 1 ul
25 uM		0.25% DMSO ; 0.5 ul
12.5 uM		NO DMSO

Fixing and permeabilizing

After 24 hours of treatment the cells were fixed with 4% paraformaldehyde (PFA) for 30 mins at room temperature followed by permeabilization with 80% ethanol. The cells were subjected to 3 consecutive washes of 1X PBS for 5 mins (100 µl) in between fixing and permeabilizing steps. The cells were stored for overnight at 4°C.

Hybridization

Following day, cells were dosed with the pre-hybridization and hybridization solution containing 40% formamide (4 ml) , 10% 20X saline sodium citrate (SSC) (1 ml) , 60% DEPC water (5 ml) , bovine serum albumin (BSA) (20 µl) , single stranded DNA (100 µl) , RNase inhibitor VAC (10 µl) , and complementary DM oligonucleotide Cy3 CAG for DM1 and Cy3 CAGG for DM2 (1 µl of 100ng/ul of probe for every 2 ml of hybridization solution). Cells were incubated overnight at 37°C.

Hoechst Staining

Cells were washed x3 with 1% MgCl₂ (PBS+MgCl₂) and were further stained with 0.05 % Hoechst stain (10 mM) (5 µl in 10 ml) for 20 minutes at room temperature. The plate was then stored at 4°C with 1M MgCl₂ + PBS until imaged.

3.2.2 Imaging with ZEISS cell discoverer

The high content images were captured using a Zeiss Cell Discoverer 7 microscope at 20X (0.95) objective lens. Two wavelength channels were applied; Cy3 Foci and Hoechst DAPI. The acquisition was adjusted to identify the nuclei of cells and the foci spots in them.

3.2.3 FISH data Analysis

Nuclei and the foci quantification was achieved using software Zen blue. Data calculations and averaging was performed using Excel. P value tests, EC50 value and graphs were executed using GraphPad Prism.

3.3 CDK12 treatment on HSALR Mouse

3.3.1 Cryostat tissue sectioning

The HSALR mouse model was used. Mice were injected with CDK12 inhibitors Dinaciclib and 8081 with their respective controls. Gastrocnemius muscle tissue was sectioned using a cryostat to test the effects of treatment on the foci via immunofluorescence imaging. The table below includes the HSALR mouse sample list.

The muscle tissues were cut using the cryostat machine set at temperature -22°C and object temperature at -20°C (if the temperature is too cold, the sections will wrinkle on stage). The tissues were mounted onto a chuck and were fixed to it using optimal cutting temperature solution (OCT). The chuck was then placed in the holder and 10 μm sized sections were sliced using a blade with an angle set at 4° . Two sections per sample were obtained and the slides were labelled and stored at -80°C until immunofluorescence was performed.

TREATMENT	SEX	NUMBER OF SAMPLES
Dinaciclib	male	3
VD control	male	3
Dinaciclib	female	3
VD control	female	3
8081	male	3
V80 control	male	3
8081	female	3
V80 control	female	3

3.3.2 Fluorescence in-situ hybridization for slides

Fixing and Permeabilizing

The sections were fixed by submerging the slides in autoclaved coplin jars containing 2% paraformaldehyde (PFA) diluted in PBS for 30 minutes at 4°C . After a 1X PBS wash the slides were permeabilized in 2% acetone for 5 mins at 4°C .

Hybridization

The slides were subjected to two 1X PBS washes before pre-hybridization made of 30% formamide and 70% 2 X saline sodium citrate (SSC) was splashed onto the sections at room temperature for 10 minutes. The sections were then treated with a hybridization solution [30% Formamide (3 ml) , 70% 2 X SSC (7 ml) , 0.2 X bovine serum albumin (1µl) , 0.2 X Rnase inhibitor VAC (1 µl) , 10mg/ml Yeast tRNA (100 µl) and 500ng/µl complementary DM oligonucleotide Cy3 CAG (0.5 µl)] and moist incubated at 42°C for 2 hours. Following hybridization, the slides were splashed with post-hybridization solution (30% formamide and 70% 2 X SSC) at 45°C for 30 minutes.

Hoechst Staining

The slides were submerged in 1 X SSC and 1 X PBS solutions at room temperature after which 3 washes of PBS+MgCl₂ for 5 minutes were performed at room temperature. The sections were then stained with Hoechst solution (5ug/ml) for 15 minutes at room temperature.

The slides were covered using cover slips (avoiding bubbles) with a mounting solution and sealed using a nail polish. The slides were stored at 4°C until imaging.

3.3.3 Imaging with ZEISS cell discoverer

The images were acquired using Zeiss Cell Discoverer 7 microscope at 25X objective in confocal. 6 different random positions of the tissues were selected to image in order to obtain a reliable result. A DAPI channel was used to identify the nucleus and a cy3 channel was set to highlight the foci in them.

3.3.4 FISH data Analysis

Background subtraction and image analysis was performed via Zeiss Arivis Pro software and Excel was used for compiling and averaging the data. Multiple T tests and graphs were executed through GraphPad prism.

3.4 Genome editing to target Protein Phosphatase 1 catalytic subunits

3.4.1 Transfection with Guide RNA/SiRNA

The SiRNAs/Guide RNAs for the catalytic subunits of Protein phosphatase 1 (PP1C) : PPP1C α , PPP1C β and PPP1C γ were prepared for transfection.

SiRNA preparation

The stock solution of SiRNA was 100uM and 1ul of each SiRNAs were added at a final working concentration of 200nM.

Guide RNA and CRISPR Cas 9

The stock solution of each guide RNA in nuclease-free buffer (1 X TE buffer) was 100uM. 100uM of guide was added to nuclease free water to bring the concentration of guide RNA to 30 pmol/ μ l. Hence, 10 μ l of 30 pmol/ μ l guide RNA was mixed with 3ul of 8.8 pmol/ul of CAS9 protein to form the RNP complex. The table below depicts the guide RNA sequences (ordered from Synthego) for the three catalytic subunits of PPP1C.

TARGET	GUIDE RNA SEQUENCE
PPP1C α	GACAGAGAACGAGATCCGCGGTCTGTGCCTGAAATCCCGGGA GATTTTTCTGAGCCAGCCCATCTTCTGAGCTGGAGGCACC CCTCAAGATCTGCGGTGAGCCCCCGGCCAGGT
PPP1C β	TGCTGGGAAGGAGAGTCTGTGCCGACAAGATGGCGGACGGGGA GCTGAACGTGGACAGCCTCATCACCCGGCTGCTGGAGGGTGAG TGCGCGCCTGGCCGCGGGACAG
PPP1C γ	TCTTGCTTCCAGGTGACATCCATGGACAATACTATGATTTGCTG CGACTTTTTGAGTACGGTGGTTTCCCACCAGAAAGCAACTACCT GTTTCTTGGGGACTATGTGGACAGGGGAAAGCAGTCATTGGAGA CGATCTGCCTCTTACTGGCCTACAAAATAAAATATCCTGAGAAT TTTTTCTTCTCAGAGGGAACCATGAATGTGCCAG

Cell Transfection

The cells were split 48 hours prior to transfection and were made sure to reach 60-80% confluency levels. The cells were washed with 1X PBS, trypsinised and resuspended in 10ml of media in the universal Container. The Cells were centrifuged in media at 1000g for 10 mins followed by a resuspension in PBS, re-centrifuged at 1000g for 10 mins. The supernatant PBS was carefully removed off the pelleted cells, and 100µl of Nucleofector solution (82ul Nucleofector + 18ul Supplement 1 solution) from basic Nucleofector kit (Amaza) for primary mammalian fibroblasts was added to the cells. Further, SiRNA/Guide RNA + Cas9 was added to the transfection solution. The cells were then transferred to a cuvette and transfected through electroporation via the Nucleofector Amaza Electroporator set at program : U23 NHDF Human Adult High Efficiency. Using the mini Pasteur pipette, the electroporated cells were then transferred from the cuvette to a T75 flask of BULLIT media (FGMTM-2 Fibroblast Growth Medium-2). The BULLIT media consists of basal Medium (500ml), insulin (0.5ml), human FGF basic protein (hFGF-B) (500ml), gentamicin sulphate-amphotericin (GA-1000) (0.5ml), and FBS (10ml). The cells were incubated for 3 to 4 days at 37°C with 5 % CO₂.

The same flask of cells were used for FISH and Protein/DNA extraction.

3.4.2 Fluorescence in-situ hybridization and Imaging

The cells were plated in the Greiner 96 well plate two days prior to fixing. Fluorescence In-situ hybridization and imaging was performed the same way as mentioned above in section 3.2 (Fixing to imaging via Zeiss cell discoverer section). Data analysis for FISH was done via Zen Blue software to compare the quantity of foci in control and transfected cells.

3.4.3 Protein Extraction

The cells were washed with 1X PBS, trypsinised and resuspended in 10ml of media in the universal Container. The Cells were centrifuged in media at 1000g for 10 mins followed by a resuspension in PBS, re-centrifuged at 1000g for 10 mins. The pelleted cells were resuspended in 1ml of PBS and transferred to an Eppendorf tube and

centrifuged for 5 minutes at 5000rpm at 4°C. Further, PBS was removed and the pelleted cells were suspended in 1X RIPA lysis buffer (50mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 1mM EDTA, 0.1% SDS) and 1µl of 100X protease cocktail inhibitor and kept under ice for the next 2 hours. After 2 hours, three cycles of freeze-thawing were performed for efficient lysis by changing the temperature of cells from -80°C to room temperature. The cells were then centrifuged at 12000rpm for 10 minutes at 4°C. The supernatant solution was then aliquoted and stored in an Eppendorf tube at -80°C until western blot.

3.4.4 Protein Estimation

The protein estimation was performed using Micro BSA protein assay kit. 5 Different concentration of BSA were made in Eppendorf tubes X2 : 40ug/ml , 20ug/ml, 10ug/ml, 5ug/ml, 2.5ug/ml, and 1ug/ml. The tubes of BSA were added with 0.5ml of working reagent. 10 ml of Working reagent : 5ml of Reagent MA, 4.8ml of Reagent MB and 0.2ml of Reagent MC. In parallel, 2 tubes of 1X PBS and two tubes of protein with 0.5ml working reagent were prepared. The tubes were incubated for 1 hour at 60°C, after which they were cooled down and used for calculating the absorbance at 562nm in the spectrophotometer. The tubes with 1X PBS were used as blanks, standard BSA tubes in pairs were used for measuring mean absorbance and the concentration of protein was calculated on the basis of its absorbance measure.

The equation used for calculating : $y = 0.0379x + 0.1012$

3.4.5 Western Blot

Sample preparation

20/30ug of extracted protein was added with 4ul of LDS sample buffer (4X) and 1.5ul of sample reducing agent (10X) in an Eppendorf. The tubes were then placed into a heating block at 95-100°C for 5 mins. The tubes were then cooled down at room temperature and subjected to a quick spin in the centrifuge.

Gel electrophoresis

The electrophoresis chamber with its reagents and gel was prepared for the experiment. 800ml of running buffer (1X) was prepared by mixing 40ml of 20X MOPS buffer (Invitrogen) with 760ml of distilled water. A 10% 1mmX10 wells Bis-Tris pre made gel (Invitrogen) was cut open and the liquid preservative was removed. The wells were then washed with the running buffer to remove any gel remaining in the wells. Further, the electrophoresis chamber was set up by introducing a dummy plate and the original plate in the chamber. The chamber was locked and filled with running buffer. The first two wells were loaded with 5ul of See Blue marker and Western Magic marker XP respectively, after which the rest wells were loaded with the prepared protein sample. The electrophoresis chamber was then selected with the program NUPAGE GEL and was allowed to run for 50 min at 200V at room temperature.

Transfer Membrane

The transfer buffer of 500ml was prepared by mixing 25ml of 20X transfer buffer, 50ml 100% methanol and 425ml of distilled water. 4 sponges and 2 filter papers were soaked in the transfer buffer in an aluminum tray. A PVDF membrane was soaked in 100% methanol for 1 min and then washed with distilled water twice (20ml). The membrane was then soaked in the transfer buffer for 5 mins. After the run was finished, the gel was taken out of the chamber and detached from the plate. The membrane soaked in transfer buffer was released on top of a gel. A sandwich was created by adding the filter paper soaked in transfer buffer on top and below of the membrane and gel respectively. The sandwich was then completed by adding 2 sponges each on top and below. The sandwich was then placed in the chamber, locked, and connected with electrodes. The inside chamber was filled with the transfer buffer while the outside was filled with distilled water. The chamber was set on the program NUPAGE BLOT and allowed to run for 60 mins at 30V at room temperature.

Immunodetection

After the run, the membrane was collected into a small tray and washed three times for 5 min each with 20ml of distilled water. The membrane was washed with 10ml of 5% powdered milk in TBST for 2-3 hours at room temperature. TBST solution : 8g NaCl, 20ml Tris-HCL (pH 7.6), 0.05% Tween 20, in 1 liter of distilled water. After 2/3 hours,

the membrane was washed in TBST three times for 5 min each at room temperature. Further the membrane was allowed to rest overnight at 4°C in 5% powdered milk in TBST containing 5-10ul of primary antibody. The next day, the membrane was allowed to rest for 30 minutes at room temperature in 5% powdered milk in TBST containing 2ul of Secondary antibody with three washes of TBST for 5 min each before and after the secondary antibody. The membrane was then placed in 3ml of Bio-Rad Clarity Western ECL Substrate detection solution (1.5ml of Peroxide solution and 1.5ml of Luminol/enhancer solution) for 5 min at room temperature.

Housekeeping Gene

The membrane was washed with TBST three times 30 mins each before it was placed in 5% powdered milk containing housekeeping gene for overnight at 4°C. The following day the membrane was again allowed to rest for 30 minutes at room temperature in 5% powdered milk in TBST containing 2ul of Secondary antibody with three washes of TBST for 5 min each before and after the secondary antibody. The membrane was then placed in 3ml of Bio-Rad Clarity Western ECL Substrate detection solution (1.5ml of Peroxide solution and 1.5ml of Luminol/enhancer solution) for 5 min at room temperature.

PROTEIN TARGET	PRIMARY ANTIBODY	SECONDARY ANTIBODY	HOUSEKEEPING GENE
PPP1C α	Mouse monoclonal PP1 α Antibody (G-4) Santa Cruz 271762	Anti-mouse IgG	Alpha tubulin
PPP1C β	Mouse monoclonal PP1 β Antibody (A-6) Santa Cruz 365678	Anti-mouse IgG	Alpha tubulin
PPP1C γ	Mouse monoclonal PP1 γ Antibody (E-4) Santa Cruz 515943	Anti-mouse IgG	Alpha tubulin

3.4.6 Western Blot analysis

The membrane was scanned under LAS-3000 MINI Scanner set at -30°C chemiluminescence for increment exposure at standard resolution. The band intensity and statical analysis was performed using ImageJ software and Excel was used for compiling and averaging the data.

3.4.7 DNA extraction

The cells were washed with 1X PBS, trypsinised and resuspended in 10ml of media in the universal Container. The Cells were centrifuged in media at 1000g for 10 mins followed by a resuspension in PBS, re-centrifuged at 1000g for 10 mins. The pelleted cells were resuspended in 200ul of PBS and transferred to an Eppendorf tube. The cells were then added with 20ul of Proteinase K and 200ul of Buffer AL and vortexed to mix thoroughly. The cells were incubated at 56°C for 10 minutes. Further, 200ul of 100% ethanol was mixed to the cells by vortex and the mixture was then pipetted into a Dneasy mini spin column placed in a 2ml collection tube. The cells were centrifuged at $\geq 6000 \times g$ (8000rpm) for 1 minute after which the collection tube was discarded. The cell containing Dneasy mini spin column was then placed into a new 2ml collection tube, 500ul of AW1 buffer was added and the cells were centrifuged for 3 mins at 20000 $\times g$ (14000rpm) . The collection tube was again discarded and the Dneasy mini spin column was transferred to a 2ml microcentrifuge tube. The DNA was eluted from the cells by adding 50ul of nuclease free water to the center of the spin column membrane. The spin column was incubated for 1 min at room temperature and further centrifuged for 1 minute at 8000rpm. The microcentrifuge tube containing extracted DNA was stored in -20°C until PCR.

3.4.8 Nanodrop

The concentration of extracted DNA was calculated using Thermo fisher's Nanodrop 2000 Spectrophotometer. 1ul of DNA samples were pipetted onto the spectrophotometer, and the concentration was calculated via the nucleic acid section in the ND 2000C software. Nuclease free water was used as blank to avoid any biased unreliable result. The concentration (ng/ml) and 260/280 ratio of the sample was measured (260/280 ratio for pure DNA is ~2.00).

3.4.9 Polymerase Chain Reaction

The PCR amplification was performed using Bio-Rad/G-storm thermocycler and all the reactions involved around 100-200ng/ul of cell DNA. The oligonucleotide primers were designed using Primer3 software, 20-40 nucleotides in length with 40-60% of GC content. The table below depicts the primer sets (ordered from Sigma Aldrich) used for each DNA samples.

PRIMER TARGET	FORWARD PRIMER SEQUENCE (5' – 3')	REVERSE PRIMER SEQUENCE (5' – 3')
PPP1C α	TTCTGAGCCAGCCCATTCTT	CTTGCCCCTGTCCACATAGT
PPP1C β	TTATTTTCCGTGGGTGCCTC	CGCTCCCAGAGACTCGTC
PPP1C γ	AGGGGAAAGCAGTCATTGGA	CTGCAGTCCTAGCTACCTGG

PCR Master mix

A 25ul of PCR master mix was prepared on ice . The solution contained 0.5ul of 10uM Forward Primer, 0.5ul of 10uM Reverse Primer, 0.5ul of 10mM dNTP mix (10ul of each : dATP, dGTP, dCTP and dTTP with 60ul of Nuclease free water) , calculated volume of DNA sample (depending on the concentration of DNA), 2.5ul of 10X standard Taq buffer and 0.125ul of Taq DNA polymerase in Nuclease free water to make up the final volume as 25ul. The solution was gently mixed and centrifuged for a quick spin after which the tubes were transferred to a PCR thermocycler machine set at a temperature 95°C.

Amplification and thermocycles

The samples were subjected to Initial Denaturation at 95°C for 3 minutes followed by 30 thermocycles. The 30 cycles included 30 seconds at 95°C, 60 seconds at annealing temperature usually between 45-68°C (based on the T_m of primer pair), and a final extension at 1minuted/kb for 5mins at 68°C. The table below includes the specific temperatures used at each step of PCR for each DNA sample.

DNA SAMPLE	DENATURATION	30 THERMOCYCLES			EXTENSION
		Denaturation	Annealing	Extension	
PPP1C α	95°C ; 3 minutes	95°C ; 30 sec	55°C ; 1 min	68°C ; 1.5 mins	68°C ; 5 mins
PPP1C β	95°C ; 3 minutes	95°C ; 30 sec	59°C ; 1 min	68°C ; 1.5 mins	68°C ; 5 mins
PPP1C γ	95°C ; 3 minutes	95°C ; 30 sec	57°C ; 1 min	68°C ; 1.5 mins	68°C ; 5 mins

Evaluation of amplified DNA via Gel Electrophoresis

1.5% Agarose gel was prepared by mixing agarose with 1X TAE buffer (the mixture was heated and mixed for a few minutes to ensure the solution became entirely transparent with no visible floating agarose particles) . The gel was then allowed to set for around 30 mins. The amplified DNA (10ul) was mixed with 1ul dye and pipetted into the wells of the chamber with first well designated to 8ul of 100bp marking ladder. The DNA was then separated using gel electrophoresis at 90V for around an hour.

3.4.10 PCR analysis

The gel was imaged using a Bio-Rad Gel Doc XR+ System. The gel was carefully positioned on the scanner, and Image Lab software was used to capture and detect the bands on the gel.

4 RESULTS AND DISCUSSION

4.1 DM1 and DM2 cells with 24 hours of Rapamycin treatment

4.1.1 Rapamycin on KB

Fluorescence in-situ hybridization (FISH) was used to determine the role of the mTOR pathway in the molecular pathology of myotonic dystrophy 1. Rapamycin, a macrolide compound that functions to inhibit the mTOR complex 1 was used. The DM1 KB fibroblasts were treated with rapamycin for 24 hours at different concentrations to observe the effects of the compound on foci and obtain an EC50 value (half maximal effective concentration) which provides a concentration of a drug to cause half of the maximum possible effect. To ensure that any kind of effect produced in the cells is due to rapamycin, DMSO was used as a negative control along with SB fibroblasts (non DM) (refer figure 4.1.1).

A substantial reduction in the number of foci per nucleus was observed in DM1 cells treated with rapamycin for 24 hours at the highest concentration of 200uM, and a significant reduction was also observed at 50uM concentration. However, DM cells at 25uM and 12.5uM failed to produce a foci reduction. No foci were present in the SB control fibroblasts. Further, KB cells with DMSO at different concentrations produced no reduction confirming the reliability of the treatment. The fluorescence microscopy images are shown in figure 4.1.1B.

Statistical analysis was performed to calculate and confirm the reduction. Using an unpaired T-test a P value was calculated, which came out to be less than 0.05 suggesting a statistically approved reduction at 200uM and 50uM concentrations in comparison to the foci count in the DMSO control. The graph shown in figure 4.1.1A represents the number of foci per nucleus at different concentrations and produces a curve depicting a reduction at 200uM but with no effect at decreasing concentration. Furthermore, an EC50 value was calculated, which came out to be ~150uM (figure 4.1.1A). Therefore, the data suggested that mTOR pathway plays a role in the pathogenesis of myotonic dystrophy 1 and that rapamycin, an mTOR inhibitor can be used as a potential therapeutic against the disease.

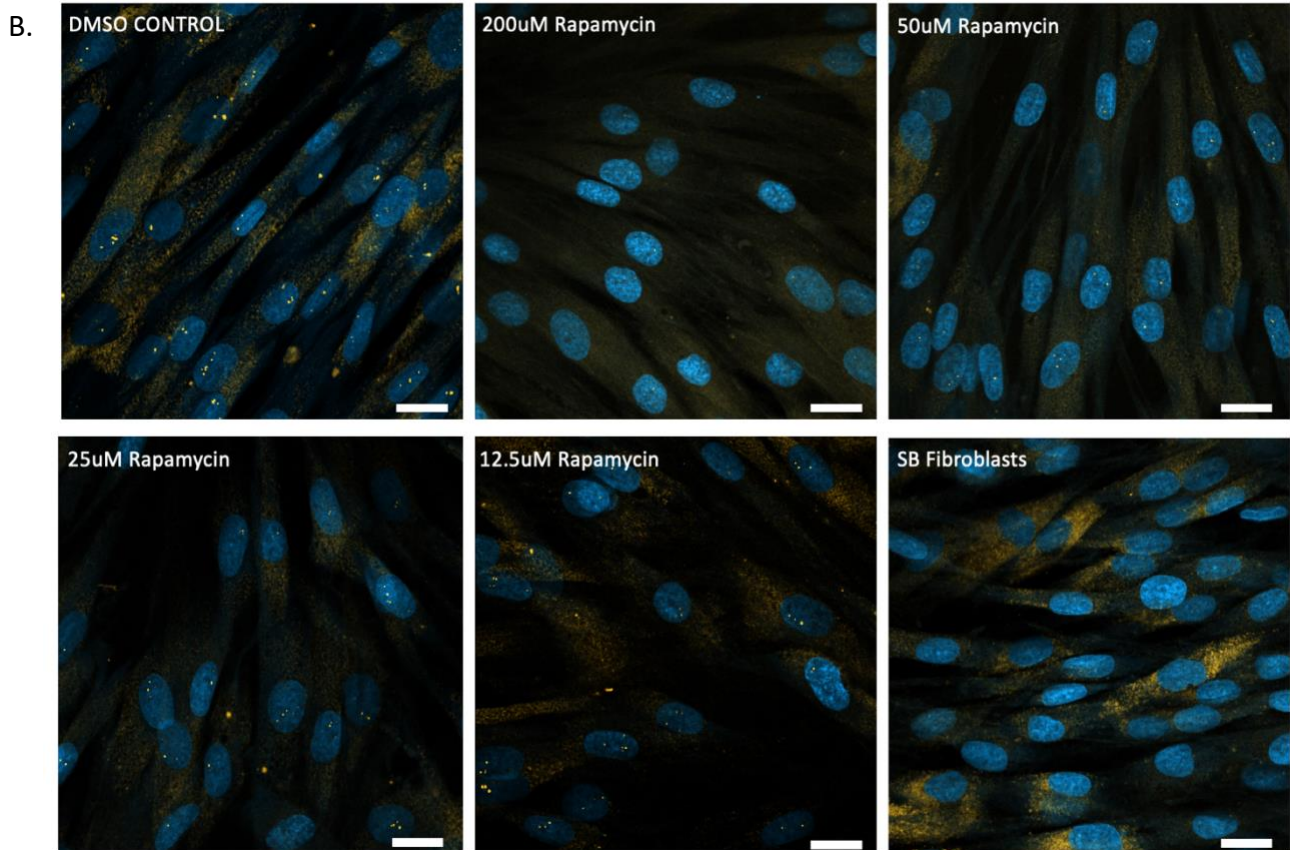
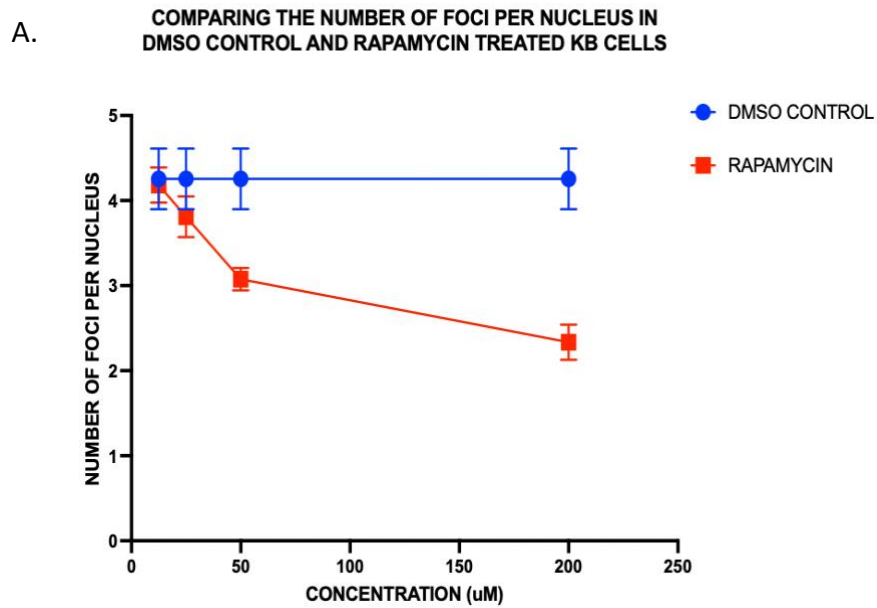


Figure 4.1.1 : Rapamycin 24 hour treatment in DM1 fibroblasts with a DMSO control.

4.1.1 (A) Graph depicts the number of foci per nucleus (n) for each concentration across the serial dilution for rapamycin treated DM1 cells versus the DMSO control (DMSO control n = 4-5; Rapamycin 200um n = 2; 50um n = 3; 25um n = 3-4; 12.5um n = 4) Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells at each concentration (P values 0.05, 0.01, 0.001) which thereby helped in calculating the half maximal effective concentration. The error bars in the graph represent the individual variations in each group.

4.1.1 (B) Representative images of the DM1 treated cells at different concentrations and control with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.1.2 Rapamycin on KAGO cells

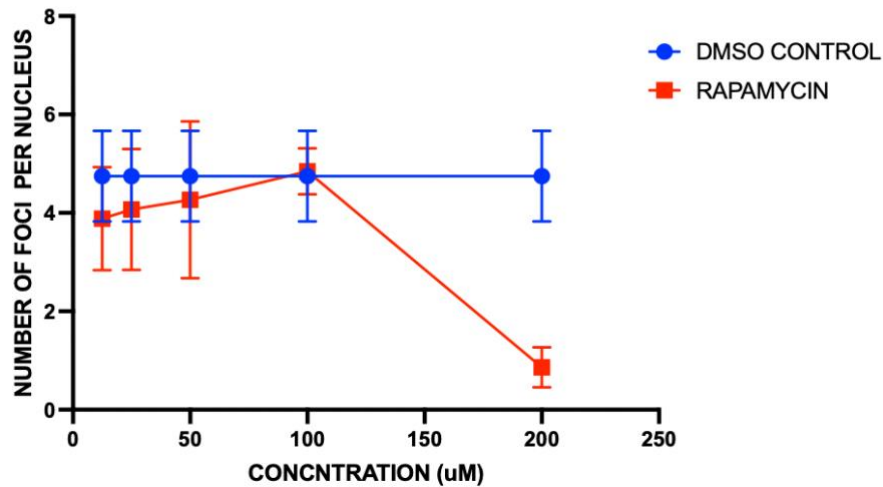
Following the effects of rapamycin in reducing the foci in DM1 cells, to examine if mTOR pathway and rapamycin play a similar role in DM2 pathology, KAGO cells were also treated with rapamycin for 24 hours at different concentrations the same way.

The fluorescence microscopy images demonstrated a significant reduction in the number of foci per nucleus in DM2 KAGO cells at the highest concentration of 200uM (fig 4.1.2B). Rapamycin at 100uM, 50uM, 25uM and 12.5uM concentrations, however failed to produce a reduction. It was also observed that the number of foci per nucleus is more in case of KAGO than KB. The average number of foci in KAGO could go up till 10 to 15 per nucleus whereas KB mostly has around 4 to 8 foci per nucleus. Similar to KB experiment, DMSO at different concentrations and SB fibroblasts were used as negative and positive control respectively to ensure the reliability of the treatment (refer figure 4.1.2)

An unpaired T-test was used to assess significance and a P value was obtained less than 0.05 in case of foci reduction at 200uM compared to DMSO control. No statistical or visual reduction was seen at any other concentrations. Thus, the calculated EC50 value came out to be ~150uM. The graph represents the number of foci per nucleus in control and treated cells and highlights the same result (figure 4.1.2A).

Even though DM1 and DM2 share a similar pathological pathway, they however have different genomic mutations and could react to therapeutics differently. It was therefore interesting to observe that mTOR pathways plays a role in both DM1 and DM2 pathogenesis.

A. **COMPARING THE NUMBER OF FOCI PER NUCLEUS IN DMSO CONTROL AND RAPAMYCIN TREATED KAGO CELLS**



B.

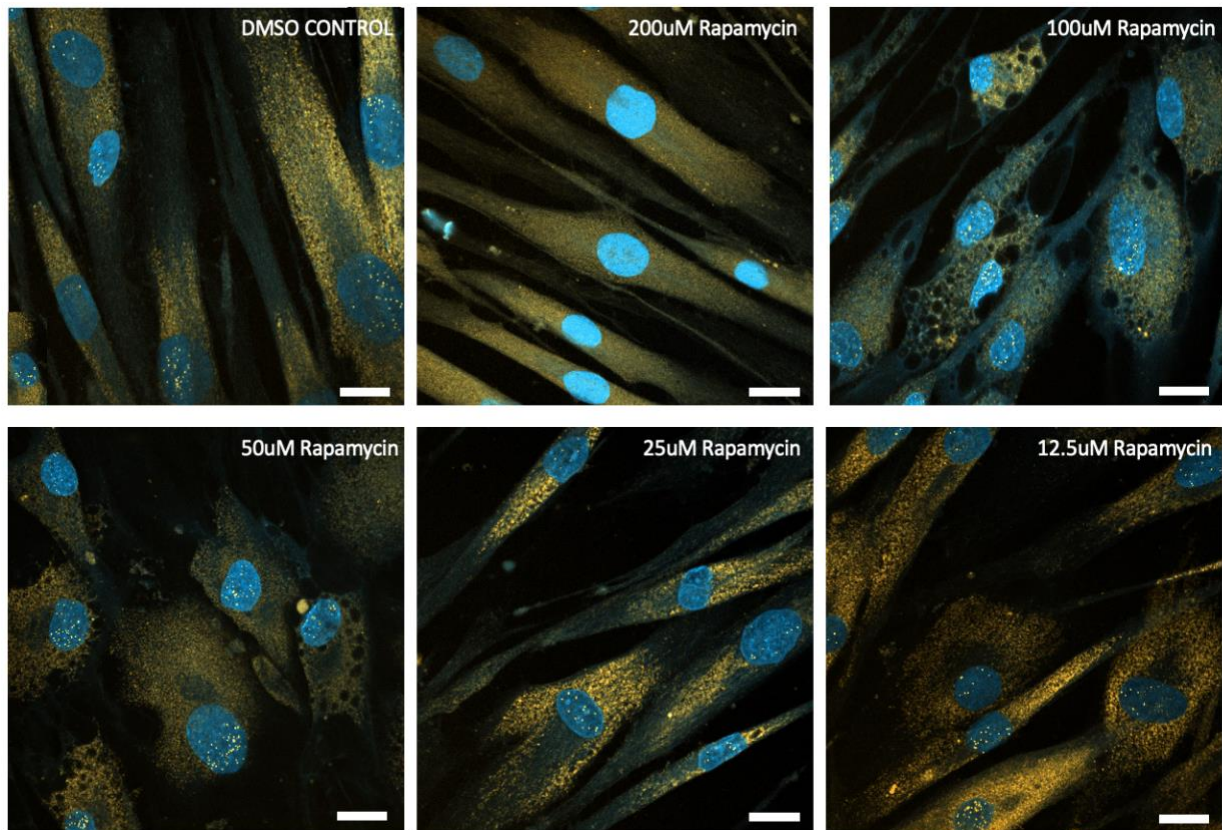


Figure 4.1.2 : Rapamycin 24 hour treatment in DM2 fibroblasts with a DMSO control.

4.1.2 (A) Graph depicts the number of foci per nucleus (n) for each concentration across the serial dilution for rapamycin treated DM2 cells verses the DMSO control. (DMSO control n = 4-5; Rapamycin 200um n = 0-1; 100um n = 4-5; 50um n = 4-5; 25um n = 4; 12.5um n = 4)Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells at each concentration (P values 0.05, 0.01, 0.001) which thereby helped in calculating the half maximal effective concentration. The error bars in the graph represent the individual variations in each group.

4.1.2 (B) Representative images of the DM2 treated cells at different concentrations and control with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.1.3 Rapamycin Discussion

To understand the effect of the mTOR inhibitor macrolide rapamycin and the role of mTOR pathway in DM1 and DM2 pathology, mutant fibroblasts were treated with rapamycin for 24 hours at several concentrations to observe any changes on the nuclear foci. Since, nuclear foci are the key molecular features of the disorder, it has been utilized as a visual biomarker. Any change in the number of foci represents the change or effect on the mutant repeat expansion.

According to the microscopy images and the data analysis, it is quite evident that rapamycin reduces the number of nuclear foci in both DM1 and DM2 fibroblasts. According to the multiple unpaired T tests performed, in case of DM1, a reduction can be seen in 200uM and 50uM concentration, giving an EC50 value of ~150uM. Whereas, on the other hand rapamycin in DM2 produces a reduction only at its highest concentration of 200uM, yet resulting in the EC50 value to be ~150uM. It is also important to acknowledge the risk of type 1 error (false-positive) and a rejection of null hypothesis for the statistical data achieved. It can hence be concluded that mTOR regulation has proved its potential as a target against the disorder.

The data presented supports the results obtained by Brockhoff et al. (39). It was shown that in case of DM1, the AMPK and mTOR complex 1 pathway along with autophagy regulation is disturbed in the skeletal muscles, but more importantly, drug targeting mTOR signalling AICAR and rapamycin when tested were successful in improving the muscle function. It has been reported that the mTOR pathway play a role during a nutrient deprived condition, has an altered expression in case of myotonic dystrophy. An imbalance between the activation of AMPK and inhibition of mTOR, thereby deregulating autophagic flux has been identified as a contributor to muscle alterations and disease (39).

The figure below (figure 4.1.3) depicts the AMPK/mTOR signalling pathway in a healthy body, a myotonic dystrophy condition and a rapamycin treated situation. In healthy muscles, the pathway is highly regulated and responds to the external and internal stimuli like nutrients, energy and more in a balanced condition. The AMPK activation and mTOR inhibition is tightly regulated thereby regulating the autophagy (39). In a nutrient

deprived situation, the mTOR gets inhibited while AMPK activates leading to autophagy. However, in case of myotonic dystrophy, the AMPK/mTOR gets imbalanced, in fact the skeletal muscle does not respond to fasting conditions, affecting the signalling system and contributing to muscle atrophy (39). Thus, the mTOR gets highly activated and AMPK gets deactivated resulting in a repressed autophagic condition. Treatment with rapamycin, the mTOR inhibitor however, helps in inhibiting the mTOR complex 1, regulates the activation of AMPK and induces autophagy. Autophagy, a process involving conserved degradation to remove faulty components via lysosome, helps in the degradation of proteins and recycle substances during cellular stress. The autophagic flux is involved in various pathophysiological events including metabolic abnormalities, cell death, growth and more and thus is helpful in repairing the damaged skeletal muscles and reversing muscle atrophy in myotonic dystrophy condition. The mechanism successfully helps in reducing myotonia and improving muscle function (39).

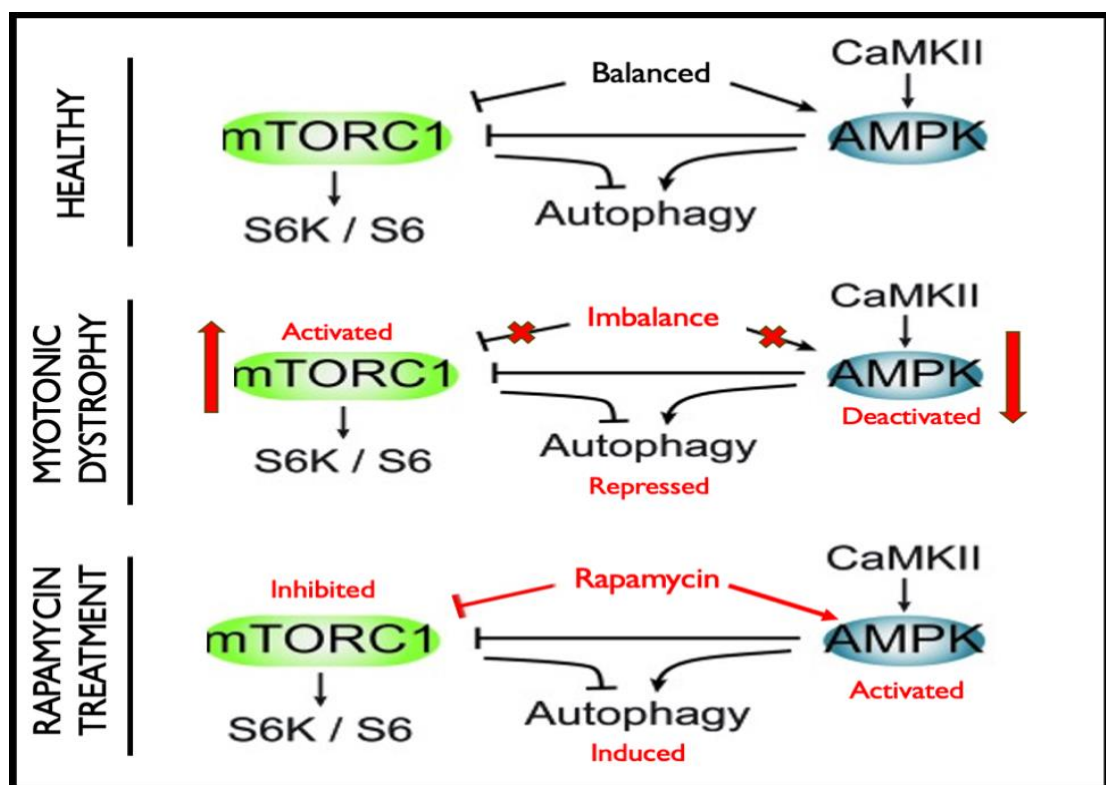


Figure 4.1.3 : Deregulation of AMPK/mTOR signaling pathway in DM1 and potential mechanism of Rapamycin in regulating the mTOR pathway. In healthy muscles, AMPK and mTOR are tightly regulated. During fasting, AMPK gets activated while mTOR gets inhibited leading to autophagy. In myotonic dystrophy conditions muscles do not respond to fasting conditions and the AMPK/mTOR pathway is deregulated with repressed autophagy. However, rapamycin, an mTOR inhibitor when used for treatment, inhibits the mTOR, activates the AMPK and induces autophagy. The signaling pathway is thus rescued by rapamycin treatment leading to reduction of myotonia (39). [Image edited and taken from (39)].

In short, the data implies that there are downstream signaling cascades that are involved in the pathogenesis of the disease and that targeting them could be a potential therapy. It is thus confirmed that mTOR does play a role in the molecular pathology of myotonic dystrophy type 1 and type 2 and could potentially be tested further. Considering, a significant result was achieved in a 24 hour rapamycin treatment, future studies could focus on a 48 hour rapamycin treatment in DM cells and observe its effects on the nuclear foci. Additionally, it could be tested in disease mouse models like HAS^{LR} mice to confirm the potential of the compound to produce any changes on the disease phenotype. The drug should therefore be tested several times on various DM cell lines and animal models for any sound conclusion to be made.

As mentioned before, DM1 and DM2 are genetically very distinct however are known to share similar molecular pathway. Previously tested macrolide compounds like azithromycin and erythromycin have produced different conclusions about their effects on type 1 and type 2 myotonic dystrophy. The macrolide data suggested its pathogenic involvement only in case of DM1 whereas in case of DM2, the azithromycin and erythromycin failed to produce any change. It was concluded that drugs may or may not be effective for both the types of DM, further suggesting that even though they share a similar pathology and clinical phenotype, it is possible that drugs helpful in treating DM1 may not be involved in DM2 (53). Since, DM2 has been clinically less known and has been overlooked due to its mild and age related symptoms, it is results like these that help in understanding of the complex pathological mechanism of diseases. It was hence, interesting to have achieve results suggesting that the mTOR pathway may have a role to play in both DM1 and DM2 and rapamycin could potentially be used for both as a compound therapy against the multisystem disorder.

4.2 HSA^{LR} mouse treatment with CDK12 inhibitors

4.2.1 HSA^{LR} mice with dinaciclib treatment

To explore the role of CDK12 in the pathophysiology of myotonic dystrophy 1, Dinaciclib, a CDK12 inhibitor with its respective vehicle control was injected into the HSA^{LR} mouse model of myotonic dystrophy 1. Mice were age and sex matched to assess the effect on nuclear foci therefore, equal number of male and female mice were injected and a vehicle control for each sample was taken (refer the table in methodology under section 3.3 CDK12 treatment on HSA^{LR} mouse). Gastrocnemius muscle was sectioned using a cryostat and tested via fluorescence in-situ hybridization technique to observe the effect of inhibitor treatment on nuclear foci. All the samples were blindly tested and analyzed blind to increase the reliability of the experiment. Additionally, it is essential to mention that no other possible cell types other than the predominant fibers were considered or identified for foci count in the tissue.

It was observed that unlike in cells the tissues contain a large number of foci gathered together (foci clusters) which are generally harder to count. The counting of the foci in the imaged tissue was conducted via Arivis software which provides a value for foci count and signals. A high background intensity was noticed in the majority of tissue sections due to which the signals received for the foci clusters are highly likely to get mixed up with signals received from the background. In order to calculate foci clusters without the background intensity hinderance, a background subtraction step was performed during the analysis through the Arivis software. The background subtraction removes the background signals received in the images to ensure the data that has been calculated is a result of foci signals only. Hence, two sets of data were inspected and compared for this experiment, data with background subtraction and data without background subtraction to reach a reliable and fair conclusion.

The images in the figure below are the representative images of the dinaciclib treated tissues along with their vehicle controls for both male and female samples (refer fig 4.2.1).

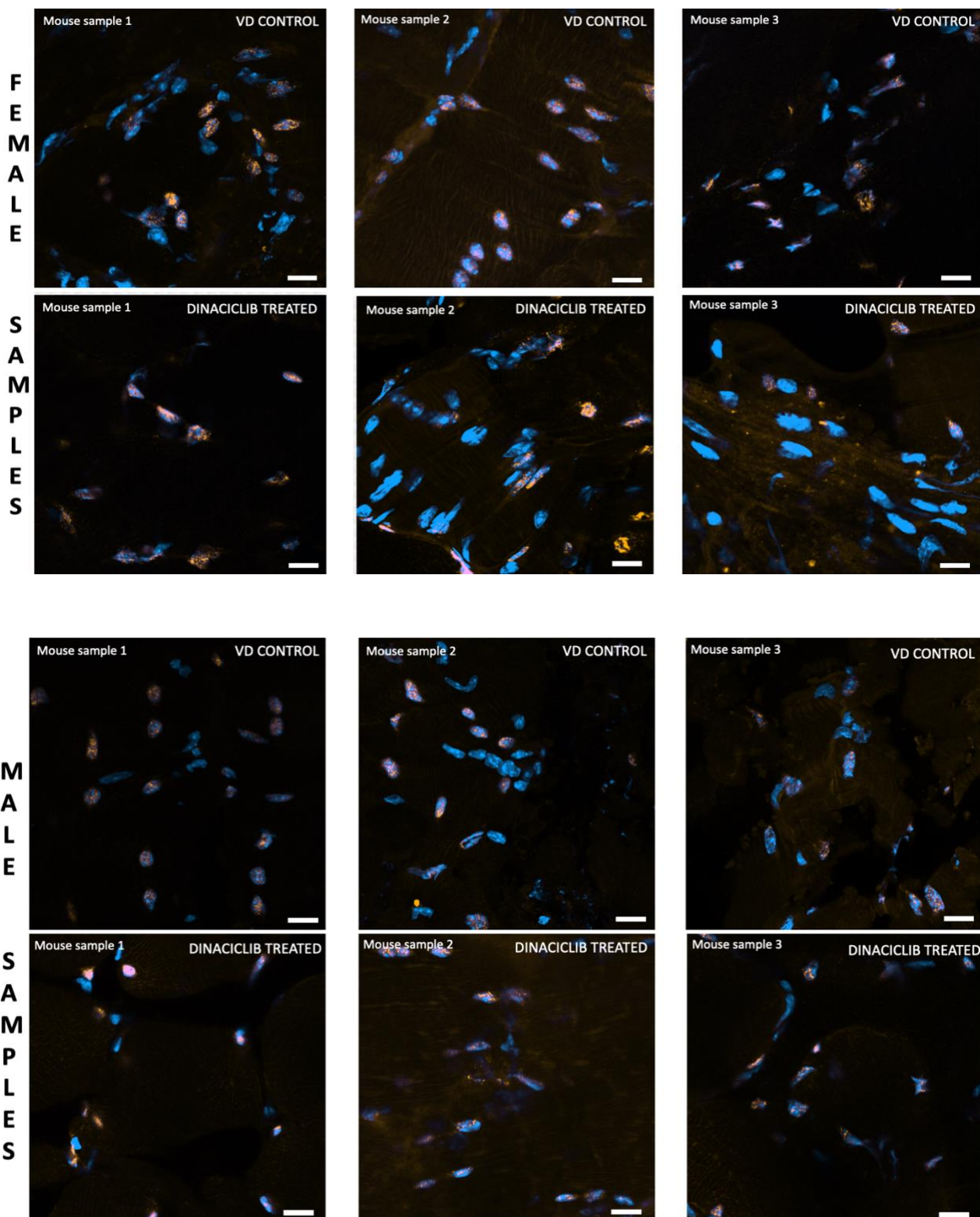


Figure 4.2.1 : Representative images of the HSA^{LR} mice gastrocnemius tissues, both male and female treated with CDK12 inhibitor dinaciclib and their respective vehicle control with the presence of foci clusters in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

Therefore, after a background subtraction, the FISH data highlighted a reduction in the total number of foci clusters present in the dinaciclib treated tissues compared to vehicle control (fig 4.2.2A). A total sum intensity count for the total foci clusters was performed which also depicted a slight decrease in the dinaciclib treated cells (fig 4.2.2B). Furthermore, the total number of foci clusters per nuclei were counted which again showcased a slight decrease in the dinaciclib treated tissues versus control (fig 4.2.2C). Lastly, another factor of comparison for the treated and control tissues was the total sum intensity of foci clusters upon total nuclei surface area which again provided a slight reduction in case of treated tissues (fig 4.2.2D). The graphs provided in the figure (fig 4.2.2) are a clear representation of the factors considered for comparison thereby highlighting the results mentioned above. However, multiple unpaired T tests were performed to confirm the significance of the reduction observed in all panels of comparison. The reduction across all panels came out statistically insignificant as the P value came out to be more than 0.05 in each case.

Thus, it can be concluded that a slight reduction was observed in all the panels of comparison for dinaciclib treatment, however, all were statistically insignificant. The background subtracted data therefore could not suggest a firm conclusion. Hence, CDK12 and its inhibition may or may not have a role to play in the molecular pathology of myotonic dystrophy 1.

Further, the data without background subtraction was also studied which highlighted a similar decrease in the total foci clusters in dinaciclib treated verses the control (fig 4.2.3A). The total sum intensity count for the total foci clusters was performed which depicted rather an increase in the dinaciclib treated cells compared to the control (fig 4.2.3B). Further, foci clusters per nucleus and total sum intensity of foci clusters upon total nuclei surface area was inspected which highlighted a negligible change produced in the dinaciclib treated verses the control (fig 4.2.3C and 4.2.3D). After unpaired T tests, it was established that no change was statically significant. No significant reduction across the panel of comparison factors were observed between treated and control tissues.

Hence, the data without background subtraction also provided no firm conclusion as statistically insignificant effects across all panels were observed (fig 4.2.3).

As mentioned above the data calculated without background subtraction is likely hindered due to high background intensity in the tissues, any conclusions made would be based on the inspection of both the data sets. Therefore, according to both the data sets (with/without background subtraction), it appears CDK12 inhibition did not help in reducing nuclear foci. It could hence not be established if CDK12 has a role to play in the molecular pathology of myotonic dystrophy 1 and if dinaciclib could be a potential target for treatment strategy. More attempts at the experiment would be required before a sound conclusion can be proposed.

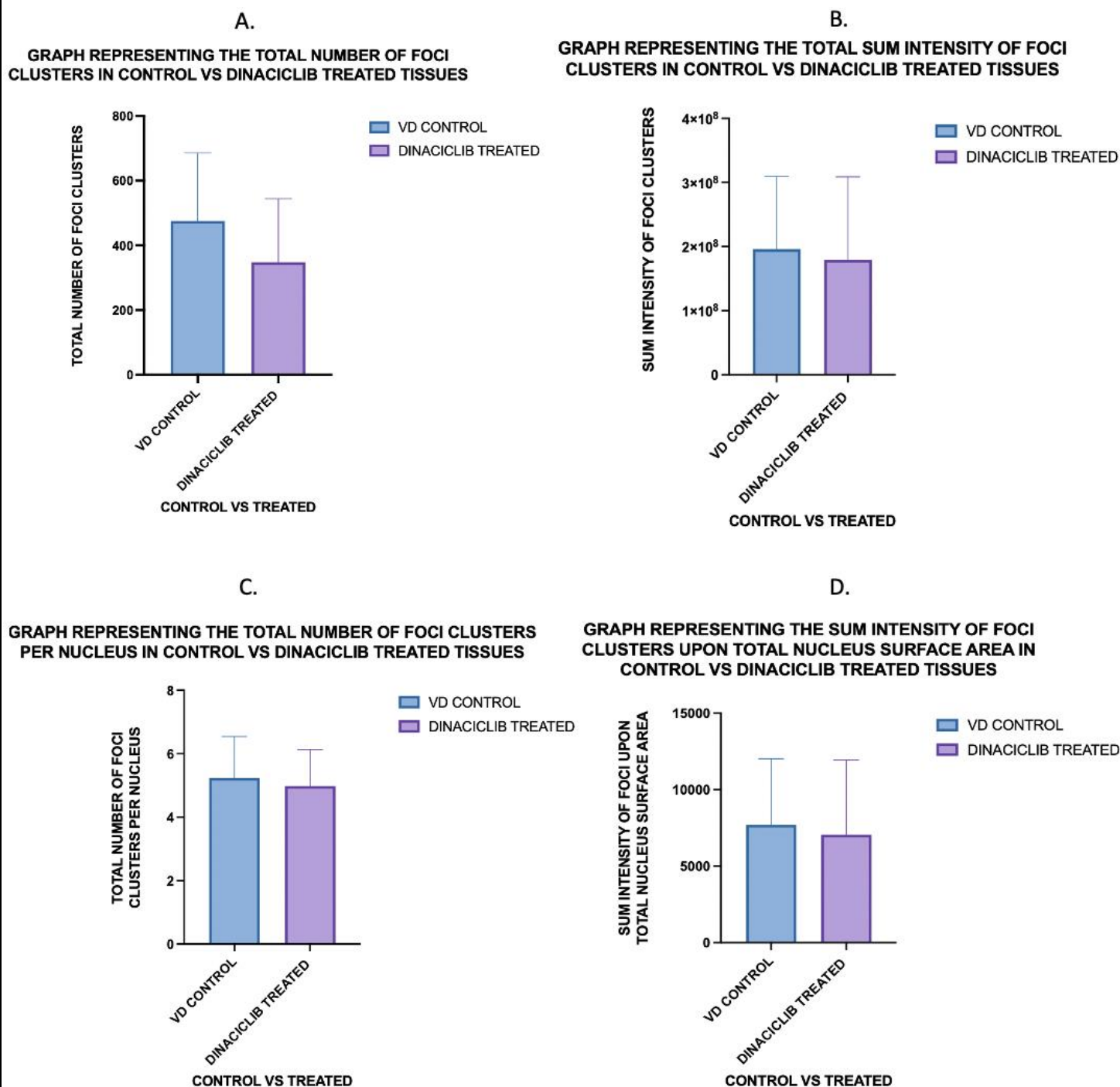


Figure 4.2.2: Graphs for CDK12 inhibitor dinaciclib treatment on HSA^{LR} mice gastrocnemius tissues with background subtraction. Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.2.2 (A) Graph depicts the total number of foci clusters in dinaciclib treated tissue verses vehicle control. (VD control n = 475; Dinaciclib treated n = 347)

4.2.2 (B) Graph depicts the total sum intensity of foci clusters in dinaciclib treated tissue verses vehicle control. (VD control n = 1.9 × 10⁸; Dinaciclib treated n = 1.7 × 10⁸)

4.2.2 (C) Graph depicts the total number of foci clusters per nucleus in dinaciclib treated tissue verses vehicle control. (VD control n = 5.2; Dinaciclib treated n = 4.9)

4.2.2 (D) Graph depicts the total sum intensity of foci clusters upon total nucleus surface area in dinaciclib treated tissue verses vehicle control. (VD control n = 7703; Dinaciclib treated n = 7054)

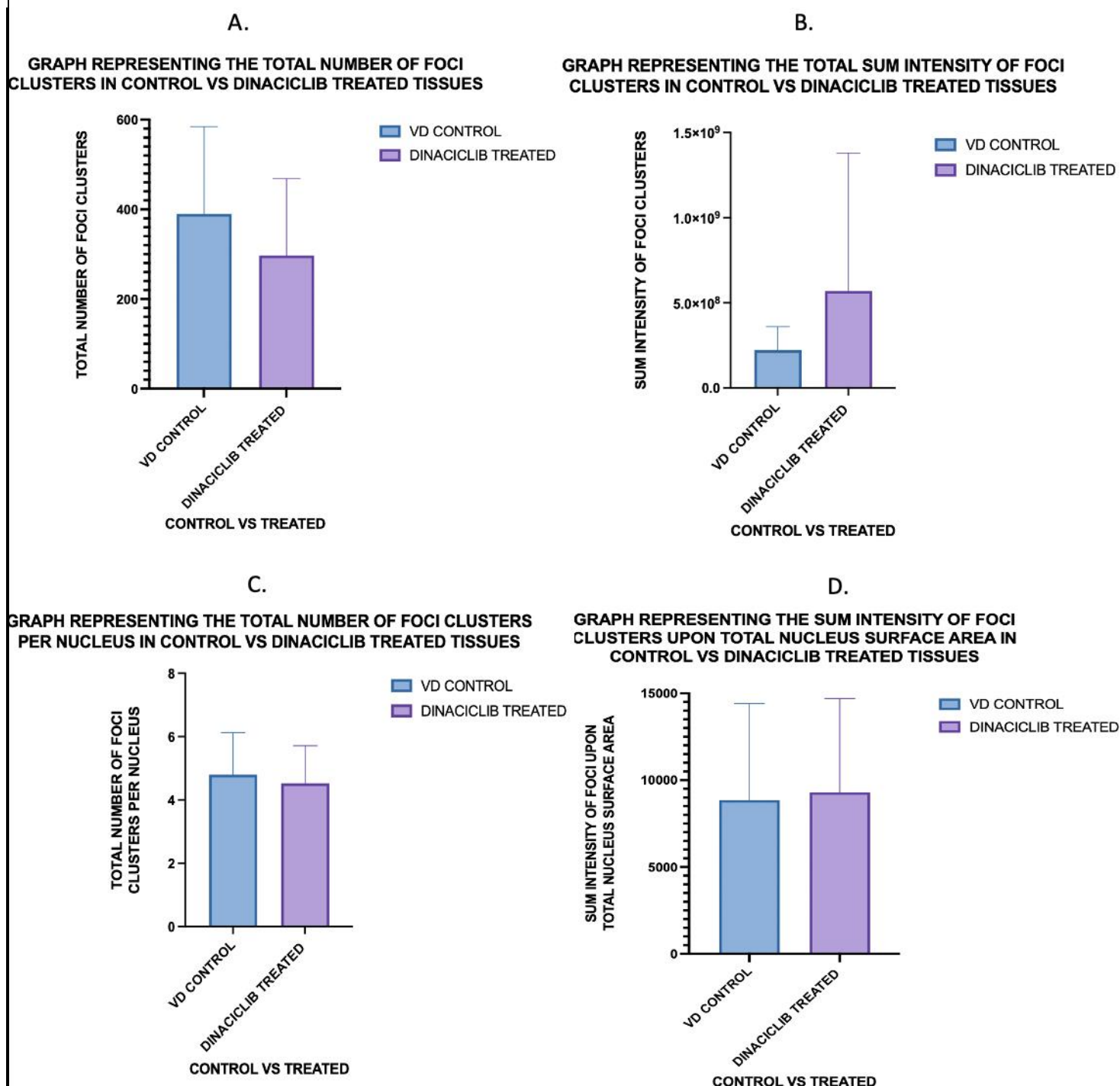


Figure 4.2.3 : Graphs for CDK12 inhibitor dinaciclib treatment on HSA^{LR} mice gastrocnemius tissues without background subtraction. Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.2.3 (A) Graph depicts the total number of foci clusters in dinaciclib treated tissue verses vehicle control. (VD control n = 390; Dinaciclib treated n = 297)

4.2.3 (B) Graph depicts the total sum intensity of foci clusters in dinaciclib treated tissue verses vehicle control. (VD control n = 2.2×10⁷; Dinaciclib treated n = 5.6×10⁷)

4.2.3 (C) Graph depicts the total number of foci clusters per nucleus in dinaciclib treated tissue verses vehicle control. (VD control n = 4.7; Dinaciclib treated n = 4.5)

4.2.3 (D) Graph depicts the total sum intensity of foci clusters upon total nucleus surface area in dinaciclib treated tissue verses vehicle control. (VD control n = 8855; Dinaciclib treated n = 9300)

4.2.2 HSA^{LR} mice with 8081 treatment

Similar to dinaciclib treatment, to explore the role of CDK12 in the pathophysiology of myotonic dystrophy 1, 8081, another newly developed CDK12 inhibitor with its respective vehicle control was injected into the HSA^{LR} mouse model of myotonic dystrophy 1. Equal numbers of male and female mice were injected and a vehicle control for each sample was taken (refer the table in methodology under section 3.3 CDK12 treatment on HSA^{LR} mouse). Gastrocnemius muscle tissues were sectioned and tested via FISH to observe the effect of treatment on nuclear foci. Similar to dinaciclib, all the samples were blindly tested and analyzed blind to increase the reliability of the experiment. Additionally, no other possible cell types other than the predominant fibers were considered or identified for foci count in the tissue.

Similar to dinaciclib experiment, a high background intensity was noticed in the majority of tissue sections due to which the signals received for the foci clusters are highly likely to get mixed up with signals received from the background. Therefore, using the Arivis software, both, background subtracted data and non-background subtracted data were calculated and compared for the analysis.

Representative images of the gastrocnemius muscle tissues of both male and female mice treated with 8081 and V80 control are depicted in the figure 4.2.4.

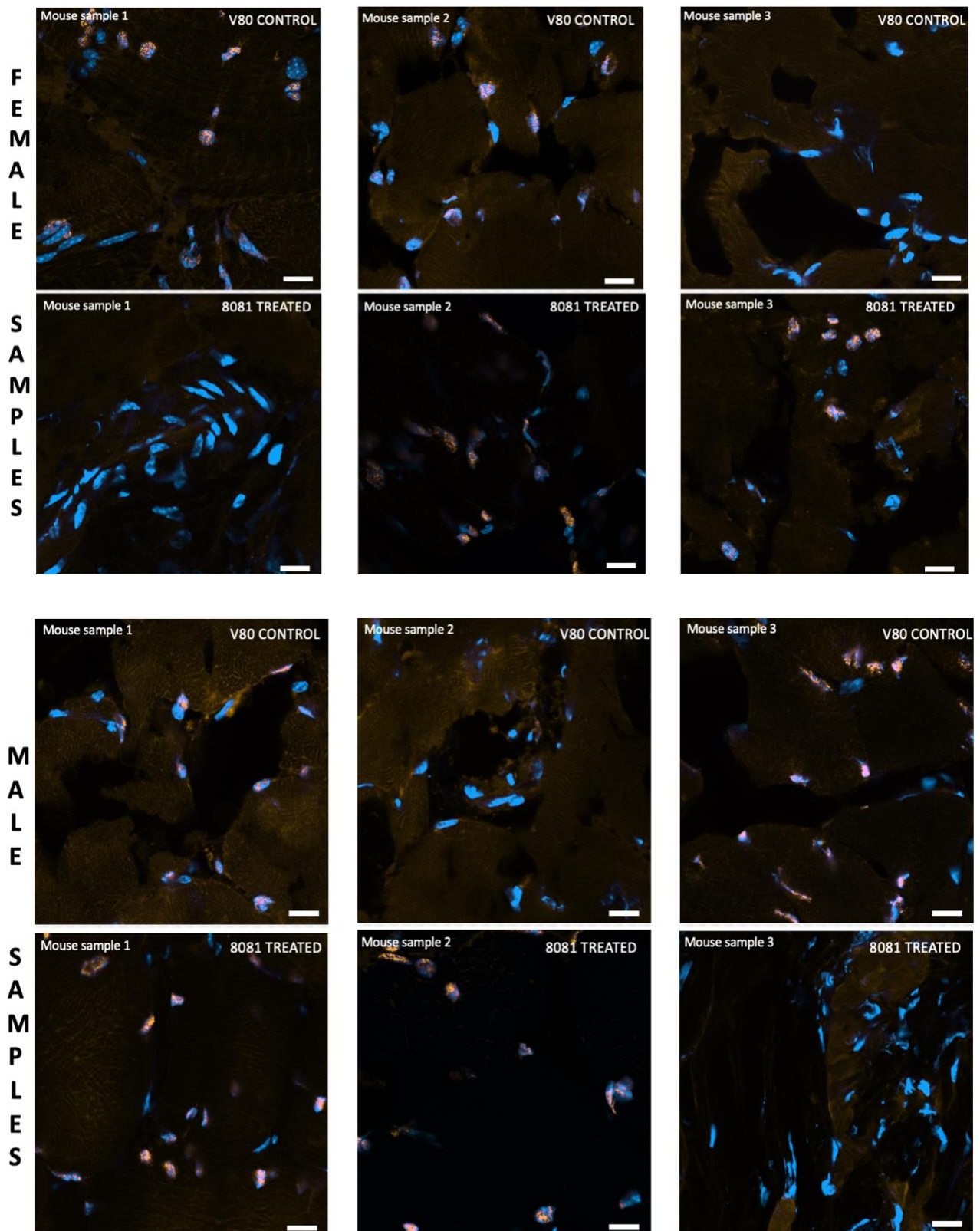


Figure 4.2.4 : Representative images of the HSA^{LR} mice gastrocnemius tissues, both male and female treated with CDK12 inhibitor 8081 and their respective vehicle control with the presence of foci clusters in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

Therefore, after a background subtraction, the FISH data highlighted a slight increase in the total number of foci clusters present in the 8081 treated tissues compared to vehicle control (fig 4.2.5A) and a slight escalation in total sum intensity count for the total foci clusters as well (fig 4.2.5B). In the case of total number of foci clusters per nuclei and the total sum intensity of foci clusters upon total nuclei surface area too, no reduction at all was seen in 8081 treated cells, in fact a negligible increase was observed (fig 4.2.5C and 4.2.5D). Statistical analysis performed also confirmed no significant change in the treated cells verses the control. The graphs in figure 4.2.5 below are a representative of the results obtained for 8081 treated tissues along with their vehicle controls (refer fig 4.2.5).

Further, the data without background subtraction was also studied which highlighted no reduction across the panel of comparison factors between 8081 treated and control tissues (refer fig 4.2.6). Multiple T tests were performed and proving the statistically insignificant data obtained.

Similar to dinaciclib experiment, any conclusions made would be based on the inspection of both the data sets for 8081 treatment.

Thus, the data suggested that 8081 failed to produce any reduction in foci clusters and may not be a potential candidate for therapeutic use against DM. Unlike dinaciclib, not even a single visual reduction was observed in case of 8081 treatment. The aim to address the role of CDK12 in the pathogenesis of myotonic dystrophy could hence not be achieved. More repeat attempts at the experiment would be essential to firmly reach a fair conclusion.

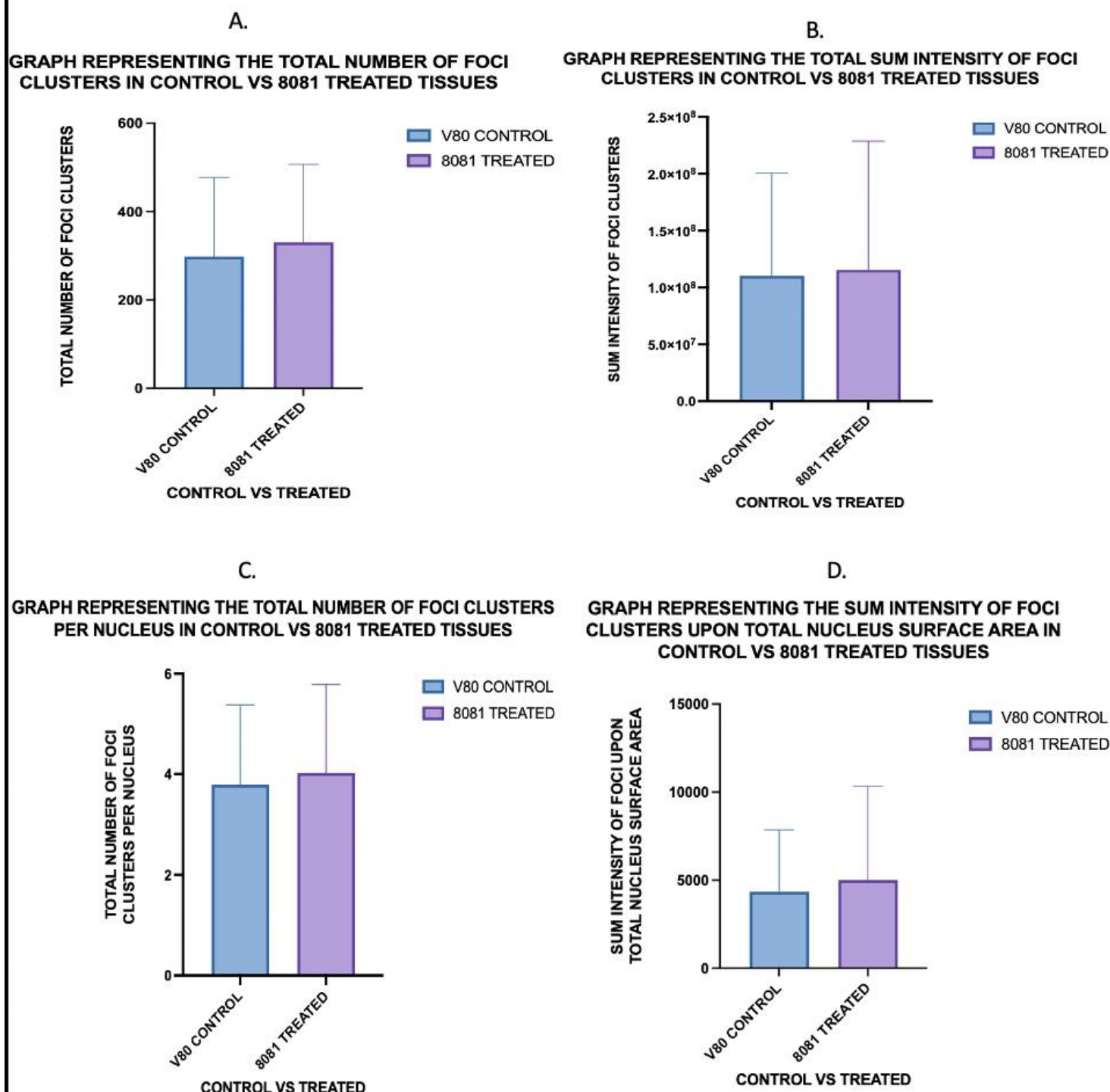


Figure 4.2.5 : Graphs for CDK12 inhibitor 8081 treatment on HSA^{LR} mice gastrocnemius tissues with background subtraction. Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.2.5 (A) Graph depicts the total number of foci clusters in 8081 treated tissue verses vehicle control. (V80 control n = 298; 8081treated n = 330)

4.2.5 (B) Graph depicts the total sum intensity of foci clusters in 8081 treated tissue verses vehicle control. (V80 control n = 1.1x10⁷; 8081treated n =1.1x10⁷)

4.2.5 (C) Graph depicts the total number of foci clusters per nucleus in 8081 treated tissue verses vehicle control. (V80 control n = 3.7; 8081 treated n = 4)

4.2.5 (D) Graph depicts the total sum intensity of foci clusters upon total nucleus surface area in 8081 treated tissue verses vehicle control. (V80 control n = 4337; 8081treated n = 5004)

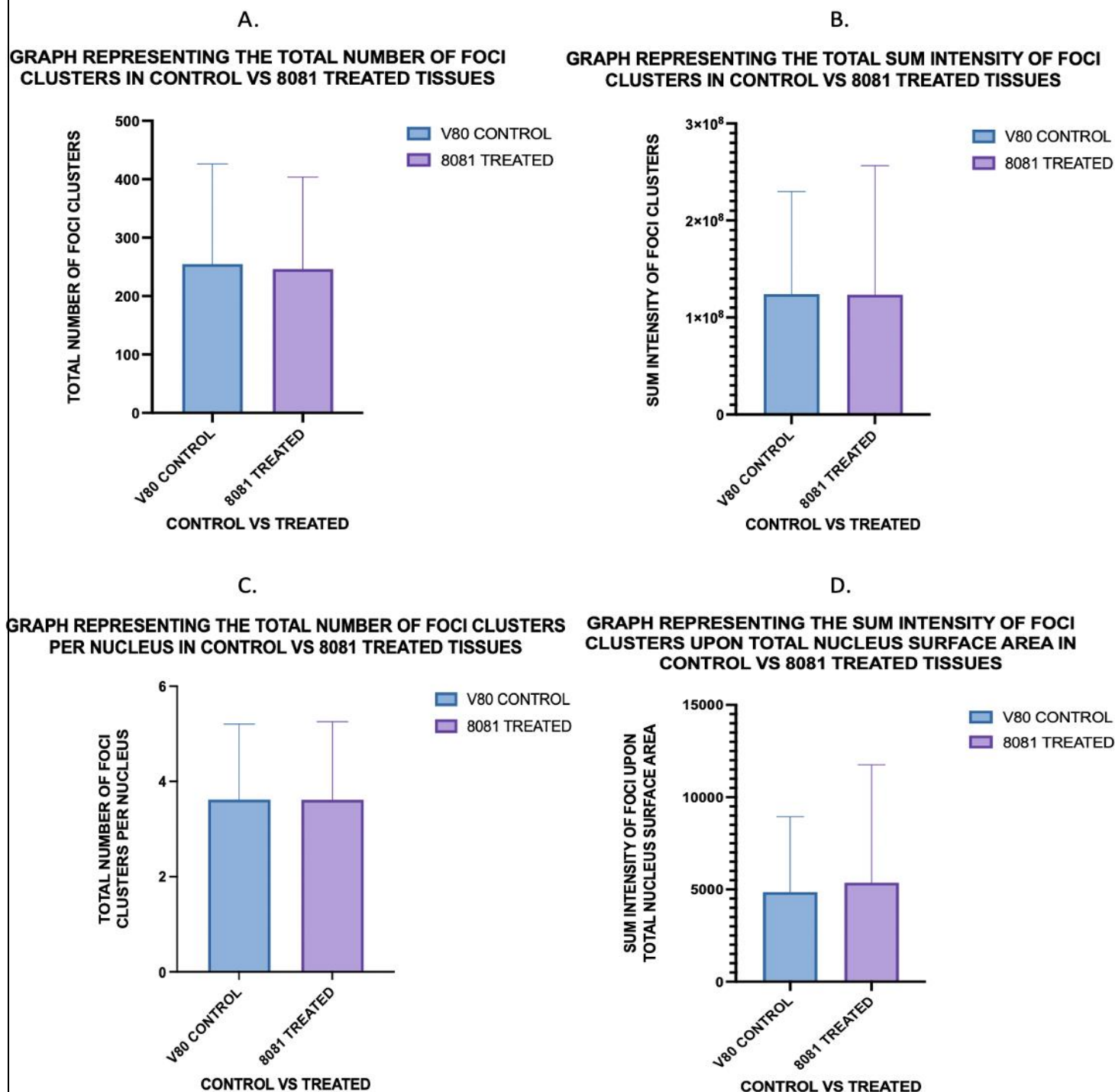


Figure 4.2.6 : Graphs for CDK12 inhibitor 8081 treatment on HSA^{LR} mice gastrocnemius tissues without background subtraction. Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and treated cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.2.6 (A) Graph depicts the total number of foci clusters in 8081 treated tissue verses vehicle control. (V80 control n = 254; 8081treated n = 246)

4.2.6 (B) Graph depicts the total sum intensity of foci clusters in 8081 treated tissue verses vehicle control. (V80 control n = 1.2×10^7 ; 8081treated n = 1.2×10^7)

4.2.6 (C) Graph depicts the total number of foci clusters per nucleus in 8081 treated tissue verses vehicle control. (V80 control n = 3.6; 8081treated n = 3.6)

4.2.6 (D) Graph depicts the total sum intensity of foci clusters upon total nucleus surface area in 8081 treated tissue verses vehicle control. (V80 control n = 4859; 8081treated n = 5367)

4.2.3 CDK12 Treatment Discussion

To determine the role of CDK12 in the molecular pathology of myotonic dystrophy 1, CDK12 inhibitors dinaciclib and 8081 were injected into HSA^{LR} mice whose generated muscle tissues were tested via immunofluorescence to observe any effects on the visual biomarker nuclear foci.

Various factors were taken into consideration while comparing the data for the CDK12 treated and vehicle control tissues and a conclusion was proposed based on the results observed from all factors. As mentioned in the results, unlike cells the tissues had a large number of foci clusters in the nucleus which are generally harder to count in comparison to a few number found in cells. The results and data of the experiment were therefore tricky in terms of counting the clusters. It is hence essential to keep in mind that all the graphs presented in the paper is a result of both, a background subtraction data (an option in Arivis software for analysis) as well as non-background subtraction data. A high background intensity was observed in majority of samples due to which a background subtraction step was performed, however, to ensure a reliable result is proposed, both the data sets were equally considered and compared.

Thus, the data obtained for dinaciclib suggested that CDK12 might have a role to play in the molecular pathogenesis of DM and that dinaciclib could be a potential therapeutic that could be utilized against the disease as a slight reduction was observed across all panels though insignificant statistically. The previous literature provided by Ketley et al. in her paper “CDK12 inhibition reduces abnormalities in cells from patients with myotonic dystrophy and in a mouse model” suggested the involvement of CDK12 in the molecular pathology of myotonic dystrophy (2). Ketley successfully demonstrated in her paper that a reduction in nuclear foci was attained when the DM1 cell line was treated with dinaciclib without the presence of cellular toxicity (2). It was also concluded that CDK inhibitors predominantly reduce the repeat containing transcript. Moreover, to ensure the dinaciclib treatment was effective, an in vivo mouse model HSA^{LR} was used. The mice were subjected to intraperitoneal injection of dinaciclib and a similar blind analysis was performed. A significant reduction was observed in the number of cells containing nuclear foci. Likewise, the repeat-containing transcript were also found to be significantly reduced (2). Further, an electromyography was also

conducted to observe if the molecular changes due to dinaciclib result in any functional improvement. An improved myotonia was observed after dinaciclib treatment which clearly indicated the potential of CDK12 inhibitor as therapy for the disease (2).

Dinaciclib that has been used in early clinical trials for cancer therapy appears to be effective towards myotonic dystrophy (2). As no statistically significant reduction could be obtained across all factors of comparison in dinaciclib treatment, repeats of the same experiment would be required to confirm if CDK12 inhibition and dinaciclib could be the potential strategy to combat the disease.

Further, CDK12 inhibitor 8081 was used and tested in the HSA^{LR} mice, however no significant reduction was observed in both background subtracted and non-background subtracted data. The data hence suggests that 8081 failed as a treatment towards the disease however more repeats of the experiment would be required to reach a fair conclusion.

However, a failed 8081 treatment does not suggest that CDK12 does not have a role to play in the pathogenesis of the disorder. Ketley et al. in her paper also demonstrated a 48% increase in the CDK12 levels in DM1 biopsies in comparison to healthy volunteer muscle biopsies, which successfully indicated the involvement of CDK12 in the pathology of the disease (2).

For every cellular activity to successfully work the balance between the kinases and the phosphatases is essential. CDK12 is a cyclic dependent kinase known to play a major role in the regulation of transcription and expression of small subset of human genes. It phosphorylates the ser2 at the CTD of RNA polymerase II thereby helping in transcription. Moreover, the CDK12 protein has an essential role to play in both adult and development phases. It also plays a critical role in mRNA processing and splicing events (41, 54).

Depletion of CDK12 therefore lead to reduced expression of predominantly long genes and the most prominent group of down regulated genes include the DNA damage response genes and the critical regulators of genomic stability. Hence, CDK12 deficient cells induce spontaneous DNA damage (41, 54).

Myotonic dystrophy is associated with toxic RNA and its downstream effects, and CDK12 is considered to play a role in the regulation of transcription and processing of mRNA, hence CDK12 inhibition is expected to prevent transcription of faulty DMPK gene and thereby rescuing its toxic effects (41). Ketley demonstrated an increase in CDK12 levels in the muscle biopsies of DM patients which might be due to the difficulty faced by the DM patients to generate the mutant RNA, thereby increasing the levels of CDK12 to help in the processing of the expanded sequence (2). Therefore, targeting CDK12 inhibition might help in the prevention of toxic RNA transcription and thereby leading to the elimination of the RNA pathogenetic mechanism characteristic of DM (41).

Hence, the insignificant results obtained in the experiment do not suggest that CDK12 is not involved in the pathological mechanism of DM. The results in fact throw a light on need to repeat the experiment to achieve a reliable conclusion.

For future emphasis, further development of more specific CDK12 inhibitors could be required to fully understand the mechanism and the functional benefit of inhibiting CDK12 for DM therapy. Additionally, a lot of difficulties were faced during this experiment which highlights the need for a better software than Arivis or a better programming script for analysis to improve the counting of the large clusters more accurately. High background intensity was also one of the problems faced during analysis and therefore some essential changes would be required for the improvement of the protocol followed for the in-situ technique for slides to ensure no non-specific binding or high background is achieved.

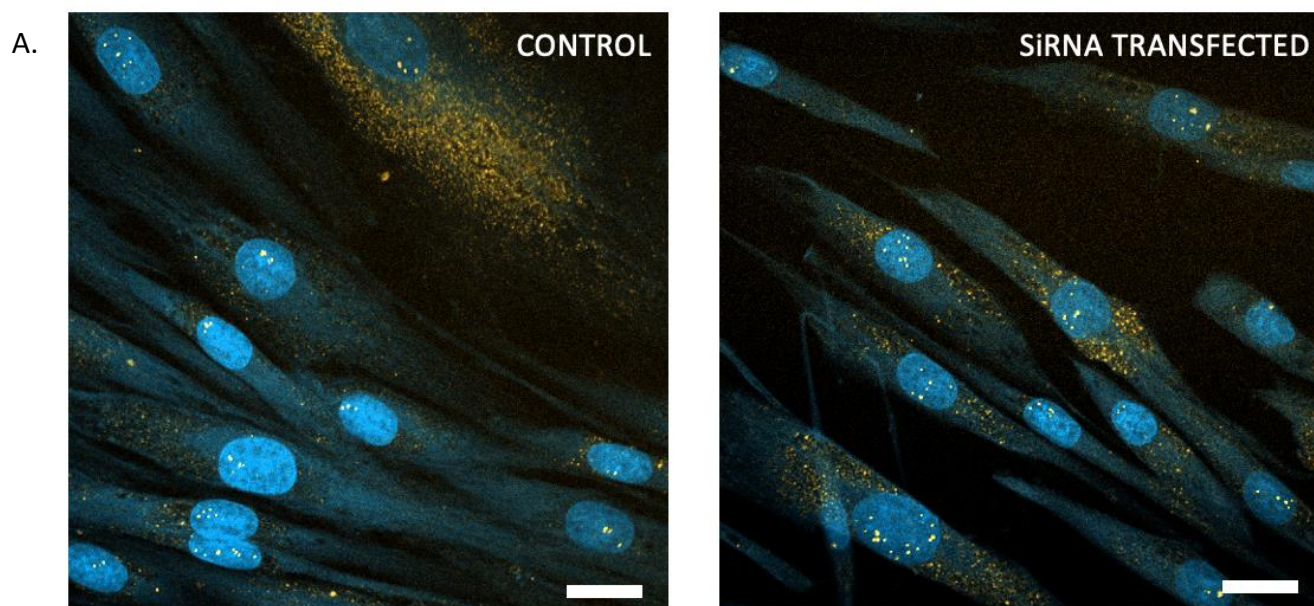
4.3 Genome editing targeting PP1 catalytic subunits

4.3.1 PP1 catalytic subunit's gene silencing via SiRNA

PP1 is a key regulator of several cellular biological processes and is known to play a crucial role in human pathologies. To investigate the role of phosphatases and the importance of phosphorylation in myotonic dystrophy, the catalytic subunits of protein phosphatase 1 were targeted. Therefore, DM1 KB fibroblasts were transfected with the siRNA targeting the three catalytic subunits of PP1 : PPP1C α , PPP1C β and PPP1C γ to observe any changes in the number of foci due to gene silencing using FISH. Additionally, protein extraction along with western blotting was performed to observe any changes in the presence of protein concentration. A scrambled siRNA was also transfected in DM1 cells which played as a negative control.

No significant reduction was observed in the siRNA transfected DM1 cells compared to the scrambled control. The fluorescence microscopy images (fig 4.3.1A) and the graph (fig 4.3.1B) depicts the same, that is no reduction in the number of foci. Statical analysis and P value tests were also performed confirming no reduction or effect. Further, PPP1C protein concentrations were tested relative to the control via western blot which was also re-probed with GAPDH to normalize any pipetting error. The results obtained from western blot (fig 4.3.1C) also suggested no change in the protein presence in siRNA transfected cells verses scramble control. Unfortunately, GAPDH protein size coincided with the size of PPP1C protein levels, alpha tubulin was hence used for further experiments to avoid the same. The obtained results therefore suggested a failed attempt at gene silencing as no change in the protein levels were observed (figure 4.3.1). Hence a result to suggest whether PP1 plays a role in the pathogenesis of DM could not be achieved. Therefore, more attempts would be needed before any reliable conclusions can be made.

The produced set of data acted as a technical data rather than a biological one and was performed majorly to confirm the previously obtained results for gene silencing in the lab. Since, gene silencing did not render any positive result, genome editing via CRISPR Cas9 targeting the three catalytic subunits individually was performed.



GRAPH REPRESENTING THE NUMBER OF FOCI IN CONTROL AND TRANSFECTED KB CELLS

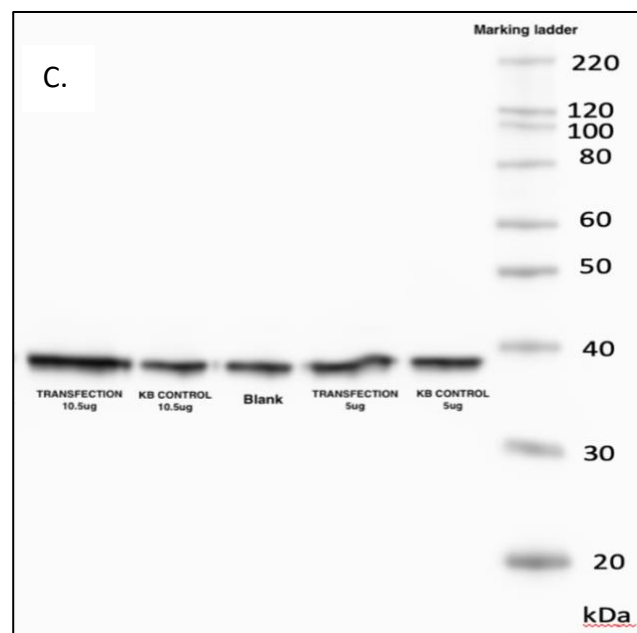
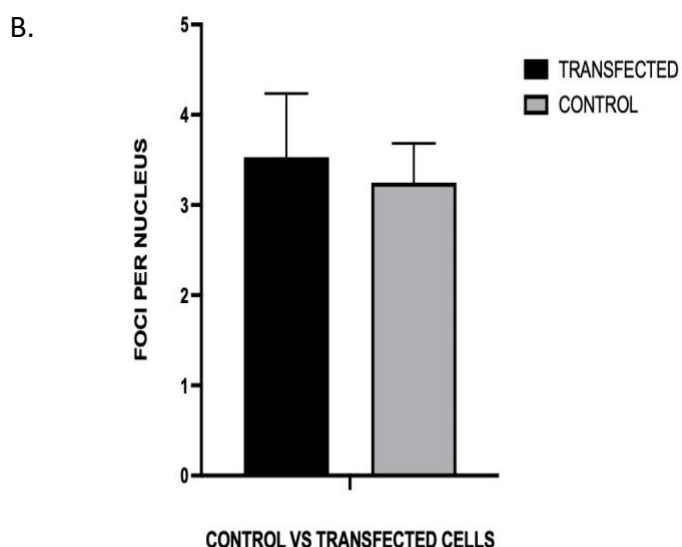


Figure 4.3.1 : Gene silencing of PPP1C with siRNA in DM1 fibroblasts with a scramble control.

4.3.1 (A) Representative images of the PPP1C siRNA infected DM1 cells and scramble control with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.3.1 (B) Graph depicts the number of foci per nucleus for PPP1C siRNA infected DM1 cells ($n=3.5$) versus the scramble control ($n=3.2$). Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and knockdown cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.3.1 (C) Western blot of PPP1C siRNA infection in DM1 cells with primary PP1 antibody and re-probing with GAPDH (Coincided bands of PPP1C and GAPDH protein sizes). The lanes from right to left show the magic marker ladder, scramble control (5ug), PPP1C knockdown (5ug), blank, scramble control (10.5ug) and PPP1C knockdown (10.5ug).

4.3.2 Genome editing via CRISPR Cas9 targeting PP1 α

Genome editing via CRISPR Cas9 targeting the PPP1C α was performed using the sequence specific Guide RNA (mentioned in methodology). The guide RNA was transfected into the DM1 fibroblasts cells through electroporation and left for 3 to 4 days. The cells were later used for foci quantification via fluorescent in situ hybridisation, protein detection through western blotting and confirmation of any gene knockout via PCR. The data and the figures below are a representative of the three biological replicates of the PPP1C α transfection along with a scrambled control. Any conclusions made are on the basis of effects produced for all three replicates. It is also essential to keep in mind that no selection or isolation of pure colony of transfected cells were conducted and the data is a result obtained from a polyclonal colony of cells which will impact the results of the experiments.

The figure below comprises the results obtained via PCR for gene knockout of PPP1C α and scramble control (fig 4.3.2A). A successful gene knockout tested through PCR would produce a band lower than the control. Thus, inspection of Fig 4.3.2A indicates no knockout as all the bands produced for the replicates are of the same size as of the scramble control. It is also possible that there is a very small deletion from the gene that could not be detected via the PCR as it migrates at a virtually identical point in the gel. Therefore, future experiments can focus on repeating the experiment and ensuring the PCR to run a little longer to observe any change.

Further, a western blot was performed by extracting the protein from the transfected and control cells. The figure below (fig 4.3.2B) includes the western blot images with the protein extracts and housekeeping gene alpha tubulin and a graph (fig 4.3.2C) to represent any effect on the protein concentration in transfected versus control cells. Even though no significant change was observed in case of PCR, the western blots depicted reduced levels of protein concentration in the transfected replicates when compared to the scramble control. PPP1C α protein levels were normalised with the alpha tubulin bands to avoid any loading inconsistencies. Despite the presence of darker bands for PPP1C α compared to the scramble control, a reduction in the proteins levels were observed after alpha tubulin was used as any inconsistencies during pipetting was

abstained. Further, statistical analysis confirmed the results observed. The graph clearly highlights the obtained low levels of protein in all the three replicates. The data therefore suggests that PPP1C α protein levels were affected through a CRISPR Cas9 PPP1C α knockout (fig 4.3.2B and 4.3.2C).

The contradictory results obtained from PCR and western blot confirms the need for a repetition of the experiment to reach any conclusion. The western blot data also explains the possibility of a short undetectable knockout in PCR as suggested above. An alternative explanation for the PCR result is that one copy of the PPP1C α gene was completely removed and the PCR primers fail to detect that copy, such that the band arises from a single intact copy.

Further, a foci quantification was performed via FISH to check if PPP1C α knockout affects the number of foci in the transfected cells compared to control. Interestingly, a decrease in the foci count was observed in PPP1C α replicate 2 and 3, PPP1C α replicate 1 however, had a slight increase of foci. According to statistical analysis PPP1C α replicate 2 and 3 have a significant reduction, however, when an average of the three replicates are calculated the reduction becomes insignificant due to the increase in the number of foci in case of replicate 1. The figure (fig 4.3.2D) includes the different sites at which scramble control and PPP1C α replicate's cells were imaged along with a graph (fig 4.3.2E) representing any effect observed on the number of foci. It is interesting to note that even though the replicates produced different results in the FISH, a majority of replicates indicated of a decrease in the foci count. It therefore suggests that PPP1C α may have a role to play in the molecular pathogenesis of myotonic dystrophy 1 however no sound conclusions can be made until the experiment is repeated and obtained with a clear result.

In short, it can be concluded that the data obtained presented very interesting results however, persistent attempts at PPP1C α gene knockout would be required before a definitive conclusion can be proposed.

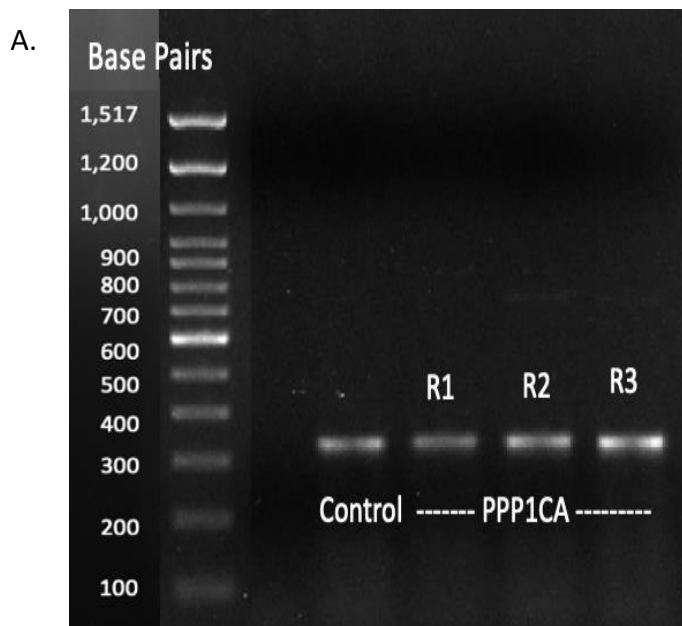
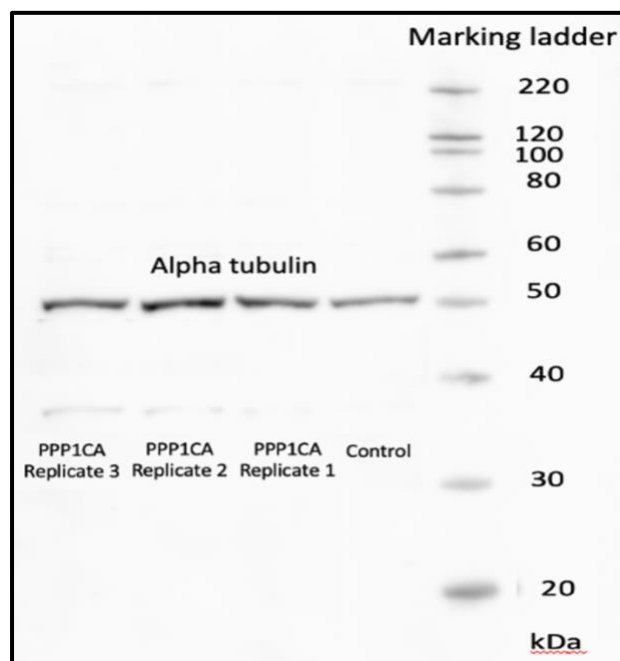
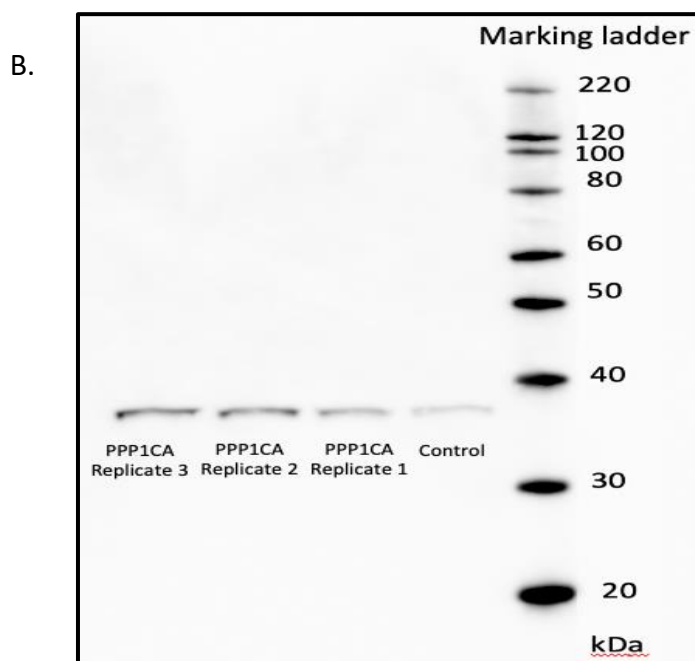


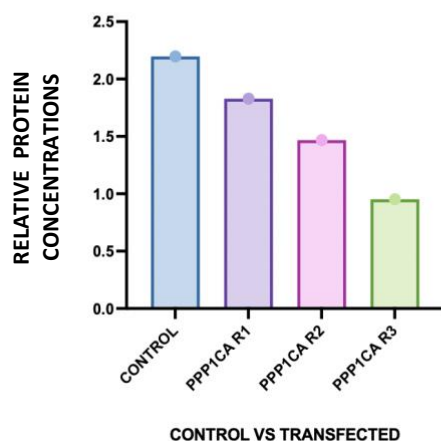
Figure 4.3.2: Three biological replicates of genetic knockout of PPP1C α in DM1 fibroblasts with a scramble control.

4.3.2 (A) PCR image of the DM1 PPP1C α knockout replicates and scramble control. The lanes from left to right show the 100 bp ladder, non-DNA sample (not visible), scramble control, PPP1C α knockout replicate 1, PPP1C α knockout replicate 2 and PPP1C α knockout replicate 3.

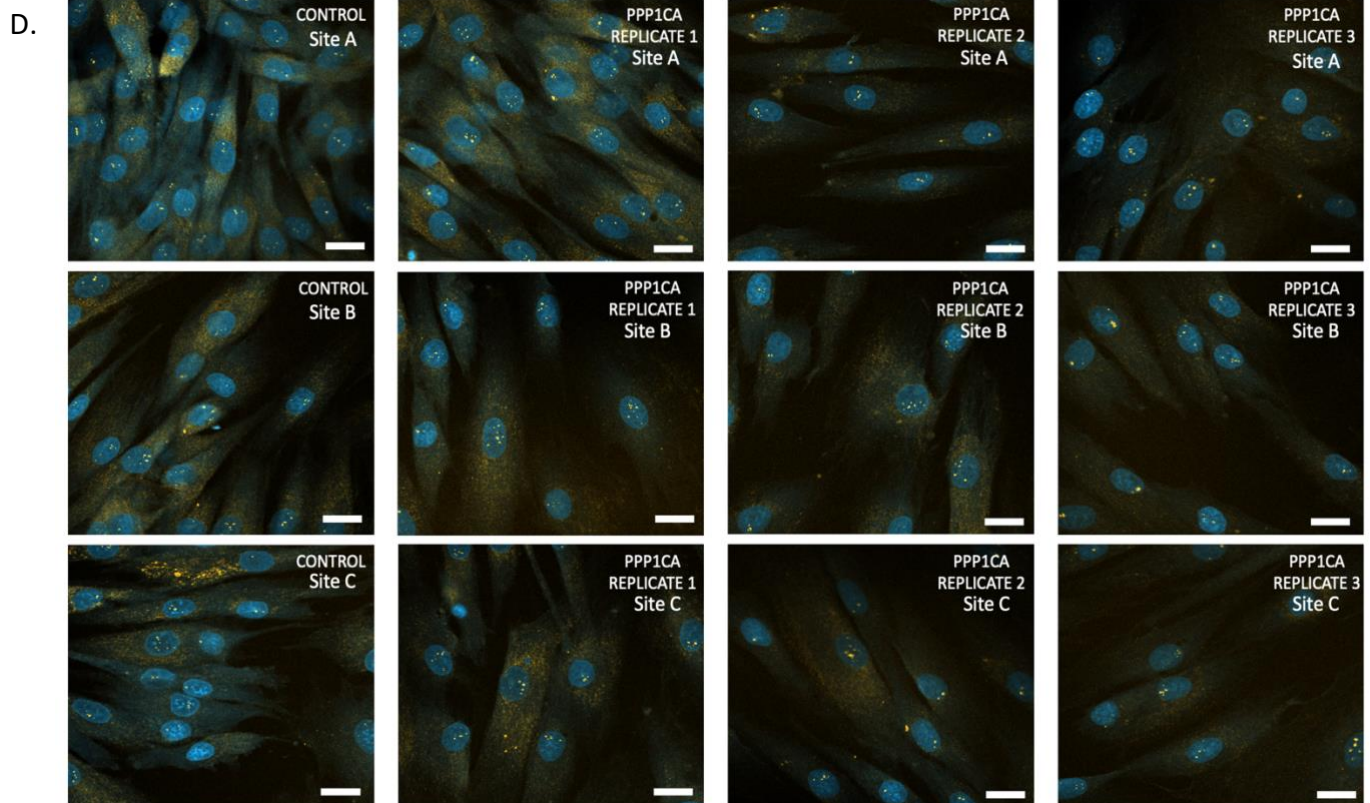
4.3.2 (B) Western blot of DM1 PPP1C α knockout replicates with scramble control and re-probing with alpha tubulin. The lanes from right to left show the magic marker ladder, scramble control, PPP1C α knockout replicate 1, PPP1C α knockout replicate 2 and PPP1C α knockout replicate 3.



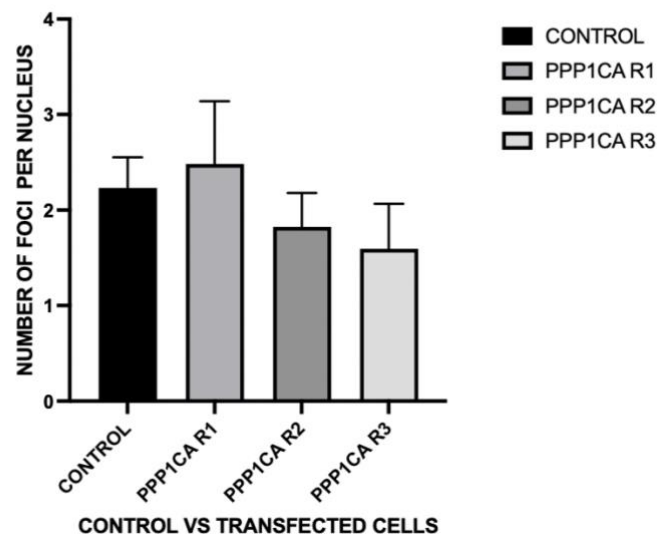
C. GRAPH REPRESENTING THE PROTEIN CONCENTRATION VALUES IN CONTROL VS PPP1C α TRANSFECTED CELLS



4.3.2 (C) Graph depicts the protein concentration values for PPP1C α knockout replicate 1, PPP1C α knockout replicate 2 and PPP1C α knockout replicate 3 relative to the scramble control. Unpaired T test was performed to determine the significance between the control and knockout cells.



E. **GRAPH REPRESENTING THE NUMBER OF FOCI IN CONTROL VS PPP1CA TRANSFECTED CELLS**



4.3.2 (D) Representative images of PPP1C α knockout replicate 1, PPP1C α knockout replicate 2 and PPP1C α knockout replicate 3 versus the scramble control at different sites with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.3.2 (E) Graph depicts the number of foci per nucleus for PPP1C α knockout replicate 1 (n=2.48), PPP1C α knockout replicate 2 (n=1.8) and PPP1C α knockout replicate 3 (n=1.5) versus the scramble control (n=2.2). Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and knockout cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.3.3 Genome editing via CRISPR Cas9 targeting PP1 β

To examine the role of catalytic subunit PPP1C β in the molecular pathogenesis of myotonic dystrophy 1, an attempt to knockout PPP1C β via CRISPR Cas9 gene editing technique and observe its counter effect on the number of foci and protein concentration was conducted. Similar to the PPP1C α knockout, PPP1C β specific guide RNA and Cas9 was transfected into the DM1 fibroblasts through electroporation and the cells were further tested via FISH, western blotting and PCR. The PPP1C β experiment also contained three biological replicates along with a scramble control and any conclusions made are on the basis of data produced for all three replicates.

The figure 4.3.3A below depicts the bands produced in PCR amplification of the PPP1C β transfected cells and scramble control. Unfortunately, no genomic knockout was obtained as all the bands were of the same size as the control. The band for PPP1C β replicate 2 was a little faint than the others due to its low DNA concentration, nevertheless, no significant change effect in PCR was observed which suggests that gene knockout could not be executed (fig 4.3.3A). It is essential to keep in mind that a polyclonal colony of cells will impact the results of the experiments.

Further, images of the western blot with and without housekeeping gene alpha tubulin along with the graph (fig 4.3.3B and 4.3.3C) representing the data for protein concentration in three replicates and control are represented. No reduction was observed in any of the three PPP1C β replicates compared to the scramble control, in fact majority of the replicates showcased an increased expression suggesting either a failed gene knockout or a lacking role of PPP1C β in the molecular pathology of myotonic dystrophy 1.

However, the data obtained from foci quantification via FISH had contradictory results as a foci reduction in all the three replicates of PPP1C β was observed. The images and the graph represent the same result. Statical analysis further confirmed the result, as the reduction observed was significant in each replicate as well as significant as an average of all (fig 4.3.3D and 4.3.3E). Hence, the data suggests that PPP1C β may have a role to play in the pathogenesis of myotonic dystrophy 1. However, as PCR and western blot suggest no significant change, it becomes important to repeat the experiment to make any sound conclusions.

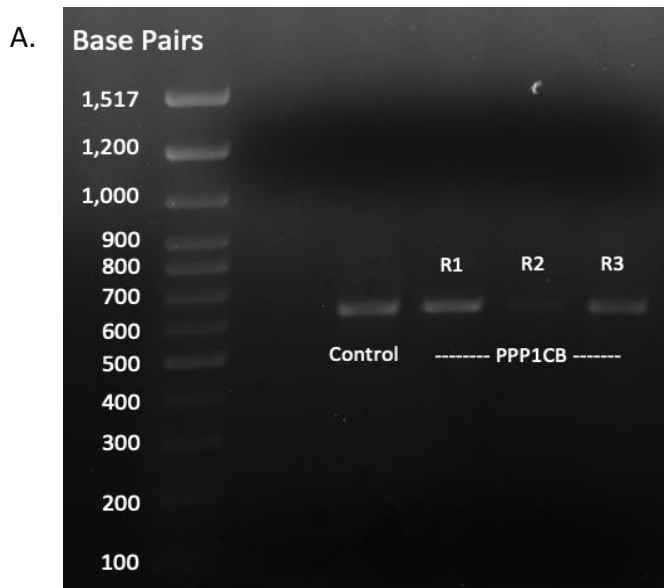
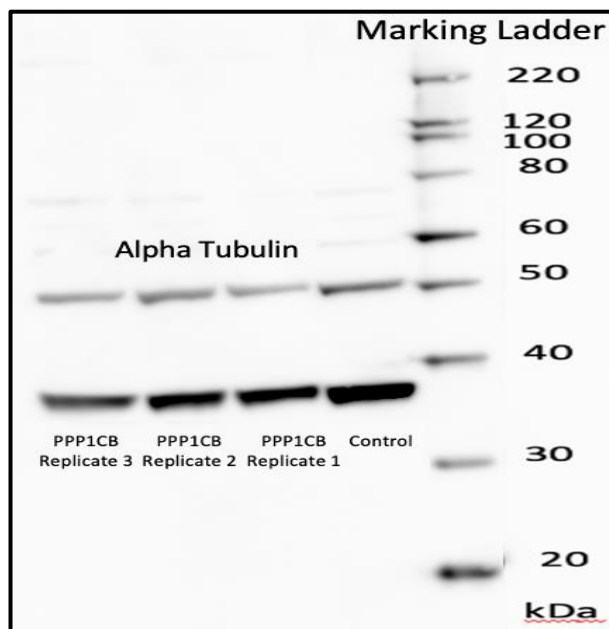
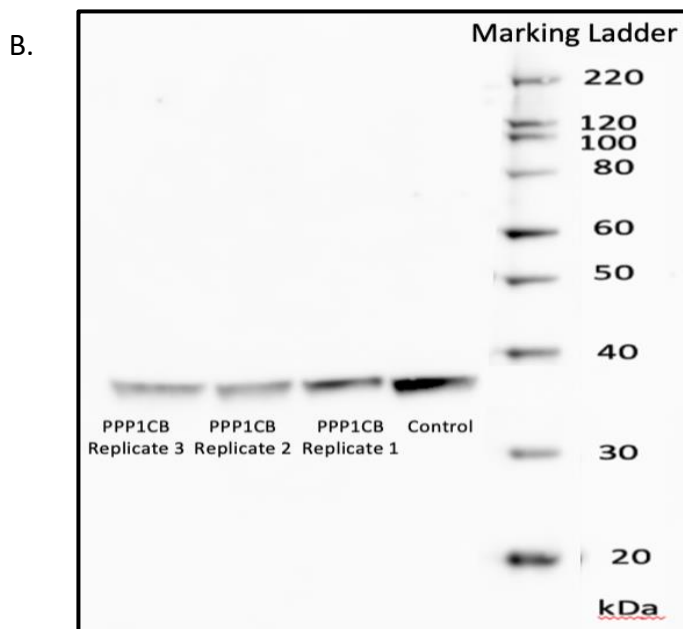


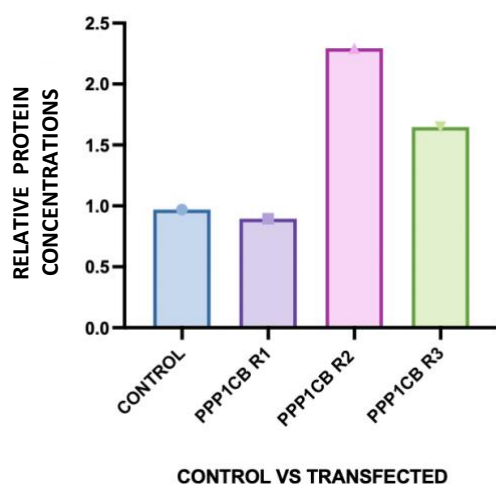
Figure 4.3.3: Three biological replicates of genetic knockout of PPP1C β in DM1 fibroblasts with a scramble control.

4.3.3 (A) PCR image of the DM1 PPP1C β knockout replicates and scramble control. The lanes from left to right show the 100 bp ladder, non-DNA sample (not visible), scramble control, PPP1C β knockout replicate 1, PPP1C β knockout replicate 2 and PPP1C β knockout replicate 3.

4.3.3 (B) Western blot of DM1 PPP1C β knockout replicates with scramble control and re-probing with alpha tubulin. The lanes from right to left show the magic marker ladder, scramble control, PPP1C β knockout replicate 1, PPP1C β knockout replicate 2 and PPP1C β knockout replicate 3.

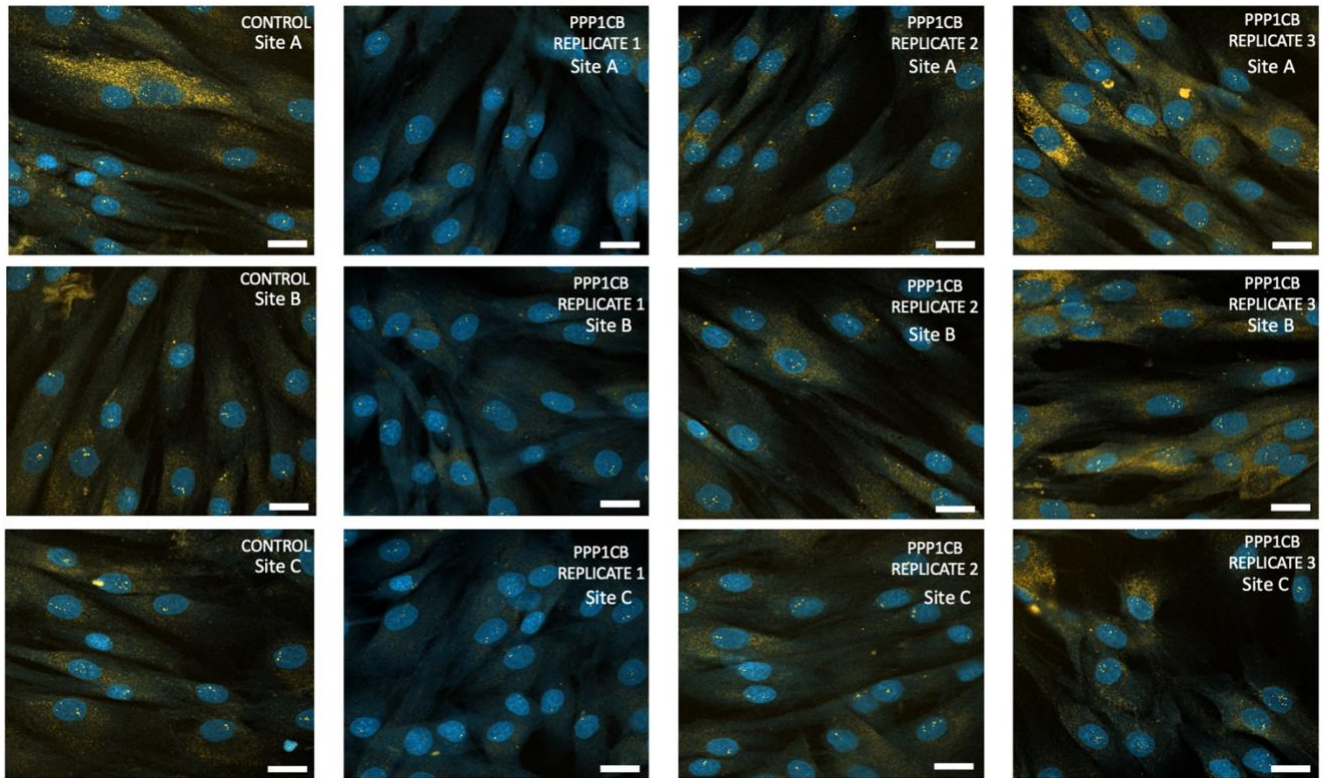


C. GRAPH REPRESENTING THE PROTEIN CONCENTRATION VALUES IN CONTROL VS PPP1C β TRANSFECTED CELLS



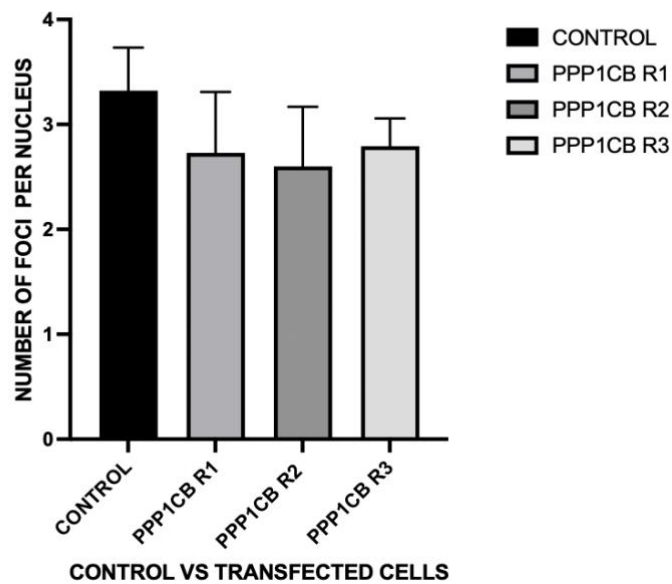
4.3.3 (C) Graph depicts the protein concentration values for PPP1C β knockout replicate 1, PPP1C β knockout replicate 2 and PPP1C β knockout replicate 3 relative to the scramble control. Unpaired T test was performed to determine the significance between the control and knockout cells.

D.



GRAPH REPRESENTING THE NUMBER OF FOCI IN CONTROL VS PPP1CB TRANSFECTED CELLS

E.



4.3.3 (D) Representative images of PPP1C β knockout replicate 1, PPP1C β knockout replicate 2 and PPP1C β knockout replicate 3 versus the scramble control at different sites with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.3.3 (E) Graph depicts the number of foci per nucleus for PPP1C β knockout replicate 1 (n=2.7), PPP1C β knockout replicate 2 (n=2.6) and PPP1C β knockout replicate 3 (n=2.7) versus the scramble control (n=3.3). Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and knockout cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.3.4 Genome editing via CRISPR Cas9 targeting PPP1 γ 2

Lastly, PPP1 γ gene knockout was conducted using CRISPR Cas9 editing technique through a PPP1 γ specific guide RNA transfected via electroporation. To identify the role of PPP1 γ in the molecular pathology of myotonic dystrophy, a foci quantification through fluorescent in situ hybridisation, effect on protein concentration through western blot and a PCR to confirm any knockout was performed in a set of three biological repeats with one scramble negative control.

The figure 4.3.4A shows the image for PCR which clearly indicated that no PPP1 γ knockout was achieved. The same sized scramble control and PPP1 γ bands for all three replicates clearly state of an unsuccessful gene knockout (fig 4.3.4A).

Validating the PCR results, the images and data from the western blot accompanied with the graph highlights of no significant reduction observed. Both, western blot with and without alpha tubulin have been presented in the figure, which after analysis depict that the PPP1 γ protein concentration in fact increased in the transfected cells than dropping. The graph highlights the failed reduction in case of all three replicates verses the control. It therefore, suggests that PPP1 γ may or may not have a part in the pathology of myotonic dystrophy 1 however repeated attempts would be required to obtain a clear result (fig 4.3.4B and 4.3.4C).

Additionally, a foci quantification in all three replicates of PPP1 γ verses control was performed which again failed to produce any foci reduction. The foci images along with the graph clearly shows that number of foci in PPP1 γ transfected cells did not reduce. Statistical analysis performed confirmed the results obtained (fig 4.3.4D and 4.3.4E).

Considering the above data, it is safe to conclude that a PPP1 γ gene knockout did not take place explaining the negative results obtained for PCR, western blot and foci count. PPP1 γ therefore may or may not have a role to play in the molecular pathology of myotonic dystrophy 1. Considering the other catalytic subunits PPP1 β and PPP1 α had some interesting results, repeat attempts at PPP1 γ knockout would also be required before proposing any conclusion.

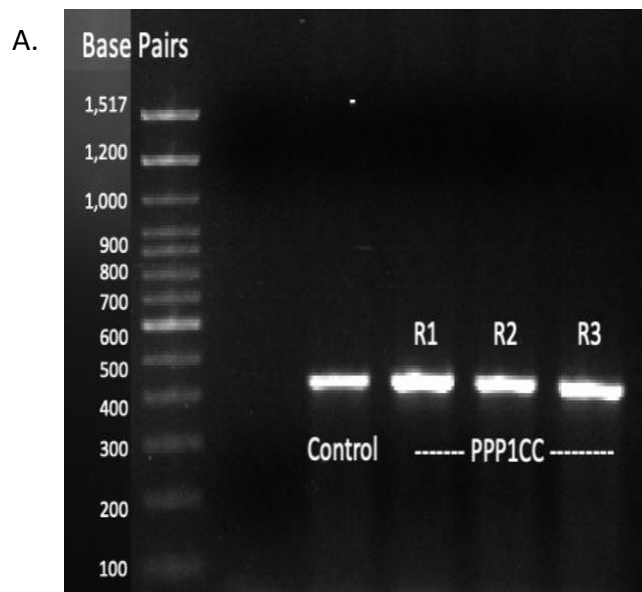
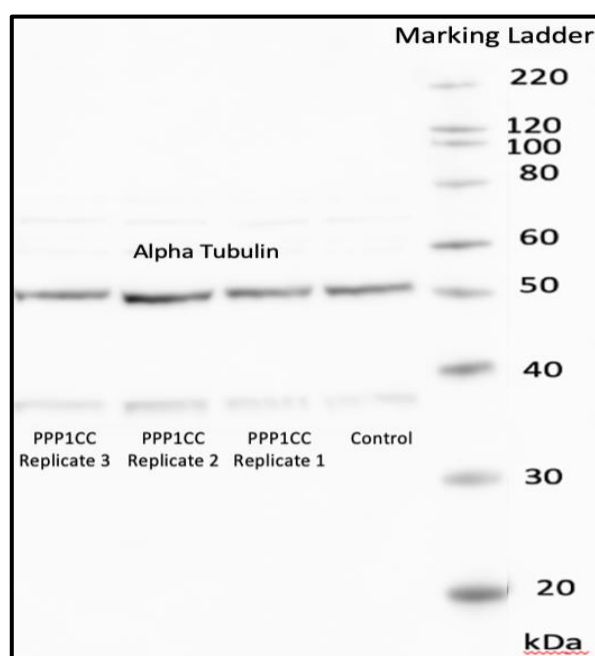
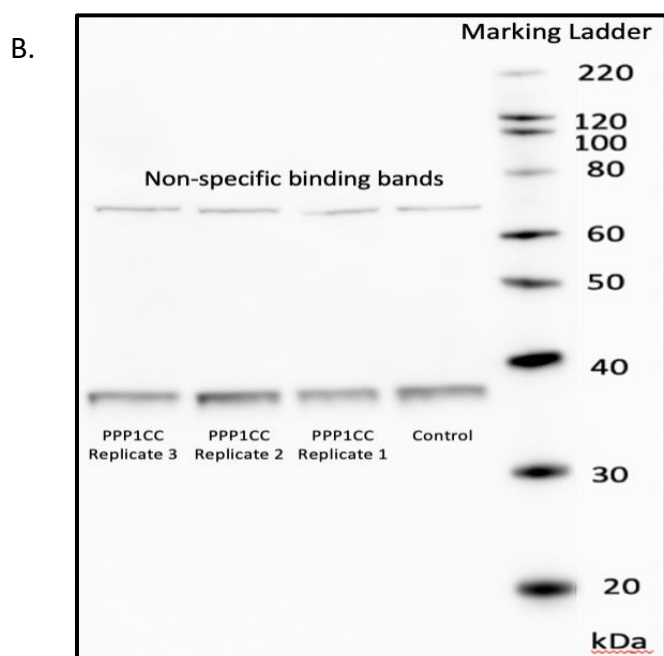


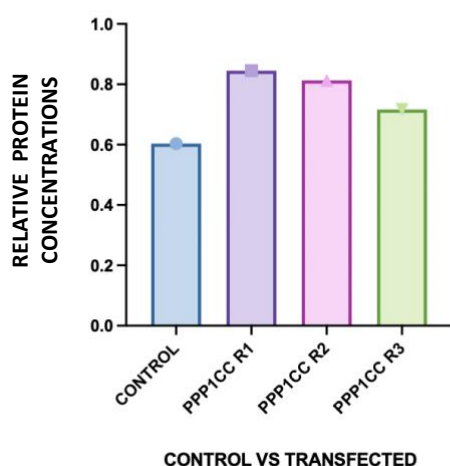
Figure 4.3.4: Three biological replicates of genetic knockout of PPP1C γ in DM1 fibroblasts with a scramble control.

4.3.4 (A) PCR image of the DM1 PPP1C γ knockout replicates and scramble control. The lanes from left to right show the 100 bp ladder, non-DNA sample (not visible), scramble control, PPP1C γ knockout replicate 1, PPP1C γ knockout replicate 2 and PPP1C γ knockout replicate 3.

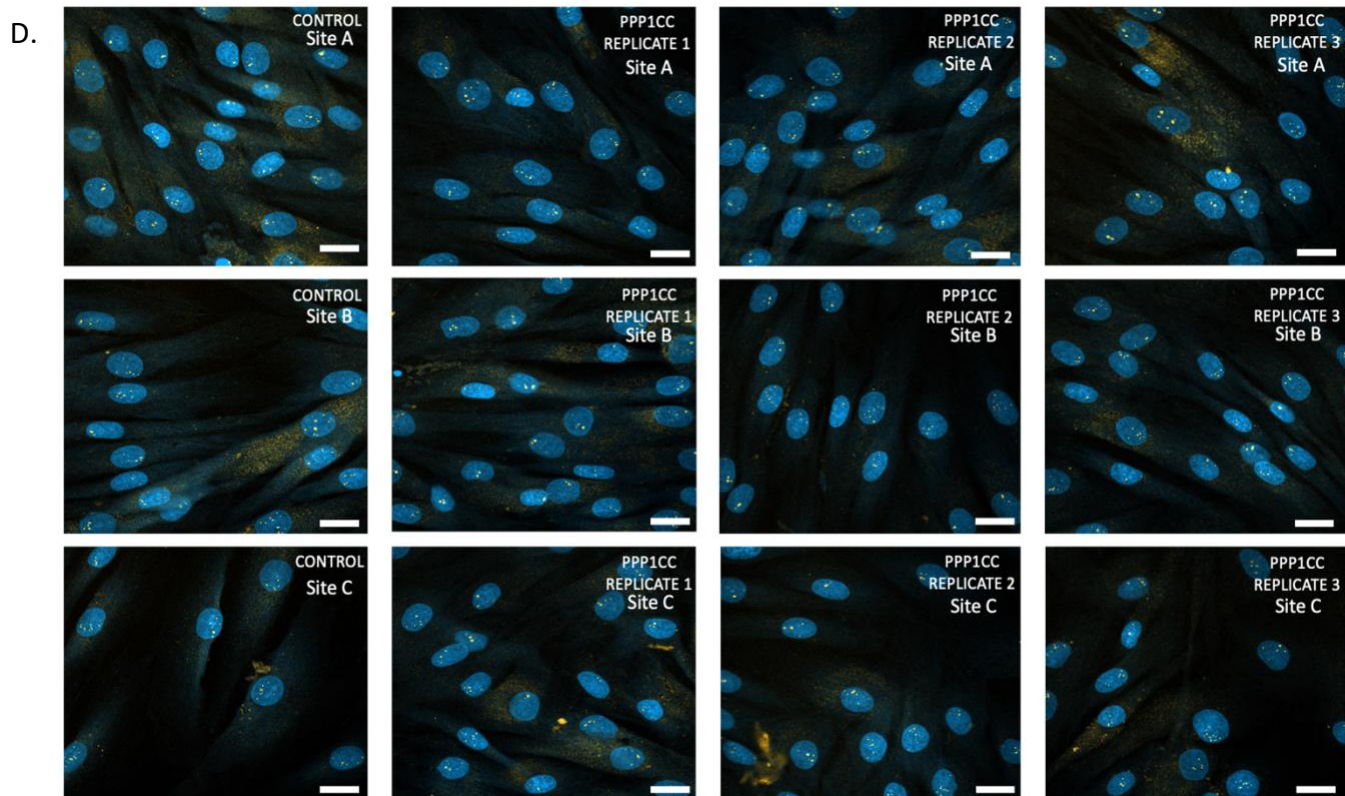
4.3.4 (B) Western blot of DM1 PPP1C γ knockout replicates with scramble control and re-probing with alpha tubulin. The lanes from right to left show the magic marker ladder, scramble control, PPP1C γ knockout replicate 1, PPP1C γ knockout replicate 2 and PPP1C γ knockout replicate 3.



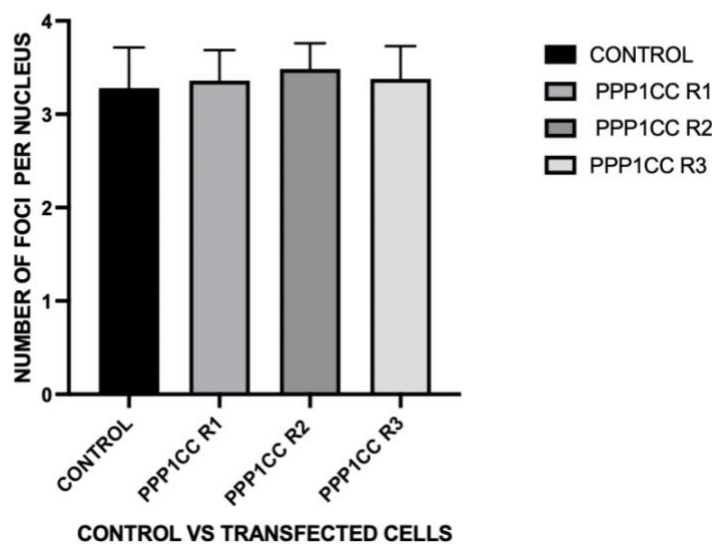
C. GRAPH REPRESENTING THE PROTEIN CONCENTRATION VALUES IN CONTROL VS PPP1CC TRANSFECTED CELLS



4.3.4 (C) Graph depicts the protein concentration values for PPP1C γ knockout replicate 1, PPP1C γ knockout replicate 2 and PPP1C γ knockout replicate 3 relative to the scramble control. Unpaired T test was performed to determine the significance between the control and knockout cells.



E. **GRAPH REPRESENTING THE NUMBER OF FOCI IN CONTROL VS PPP1CC TRANSFECTED CELLS**



4.3.4 (D) Representative images of PPP1C γ knockout replicate 1, PPP1C γ knockout replicate 2 and PPP1C γ knockout replicate 3 versus the scramble control at different sites with the presence of nuclear foci in the nucleus. The nucleus was stained with Hoechst stain (blue) while foci were probed with Cy3-CAG oligonucleotide (yellow).

4.3.4 (E) Graph depicts the number of foci per nucleus for PPP1C γ knockout replicate 1 (n=3.4), PPP1C γ knockout replicate 2 (n=3.5) and PPP1C γ knockout replicate 3 (n=3.4) versus the scramble control (n=3.3). Multiple unpaired T tests with welch corrections were performed to determine the significance between the control and knockout cells (P values 0.05, 0.01, 0.001). The error bars in the graph represent the individual variations in each group.

4.3.5 Protein phosphatases Discussion

In order to investigate the role of catalytic subunits of PP1, both gene silencing via siRNA and gene editing via CRISPR Cas9 was performed. In both the cases the DM1 fibroblasts were transfected with specific guide RNA/siRNA through electroporation and further tested via different techniques to observe effects if any. In each experiment a scramble control was present as a negative control.

Gene silencing is a molecular mechanism that suppresses the expression of a specific gene via the RNA-induced silencing complex (RISC). An siRNA is used to identify the specific RNA sequence, and binds with it to knockdown the gene thereby altering its expression. Whereas, gene editing using CRISPR is a molecular mechanism that directly modifies the specific gene. Using a specific guide RNA to identify the DNA sequence strand and Cas9 protein to cleave the strand, it carries out the process of knockout. Therefore, both gene silencing and gene editing techniques were used to knockdown/knockout PP1 catalytic subunits (55).

Phosphorylation regulation is an important post translational modification that governs most biological or cellular events. Phosphatases are a vast group of enzymes responsible for dephosphorylation of a protein. They are considered promiscuous as they contain only a handful of catalytic subunits however, they bring precision from the presence of a vast number of regulatory subunits that are the main determinants of substrate specificity. Disturbing the balance of phosphorylation can therefore alter the function of a protein and further causing the consequences (38).

In this case, disturbing the phosphorylation of PP1 may impact myotonic dystrophy 1 (38). Protein phosphatases not only engage in dephosphorylation of previously phosphorylated proteins as a regulatory mechanism, but also possess the ability to actively manage the activities of protein kinases through various direct and indirect signaling pathways. These regulatory mechanisms can occur at multiple levels including epigenetic, post-transcriptional and post-translational levels (49).

Therefore, knocking out/knocking down the catalytic subunits of PP1 suggests the elimination of dephosphorylation event of the protein. In such a case, if the gene editing technique works successfully and PP1 has a role to play in the molecular pathology of myotonic dystrophy 1, a degradation of RNA and protein levels could be observed. The figure below (fig 4.3.5) depicts how phosphorylation imbalance can cause degradation of RNA transcripts and protein levels. In the case of DM1, if PP1 is knocked out, dephosphorylation of the protein would stop which would increase the phosphorylation state of the protein. Increasing phosphorylation could therefore degrade the mRNA and protein and a reduction in foci should be expected as it is visual biomarker for myotonic dystrophy. One such potential target of PP1 is UPF1 protein, which is being tested in our lab. UPF1 is known to be involved in nonsense mediated decay, and its disbalanced phosphorylation state is expected to trigger the mRNA decay. Therefore, it may be possible that UPF1 is able to degrade the expanded transcript in myotonic dystrophy 1, when in phosphorylated state(56).

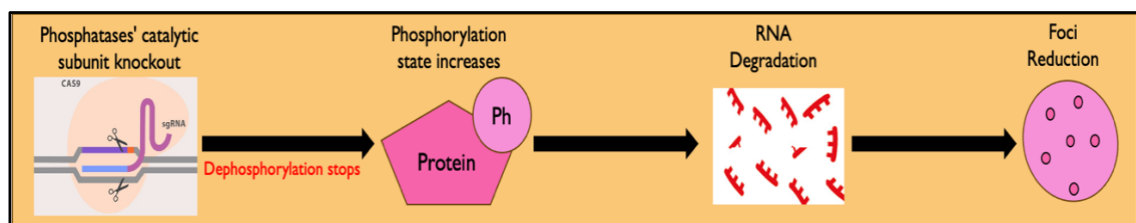


Figure 4.3.5 : Potential mechanism of knocked out Phosphatases in myotonic dystrophy. Knocking out phosphatase would eliminate dephosphorylation, resulting in increased phosphorylation state of protein. The RNA gets degraded thereby reducing the nuclear foci.

Hence, the data obtained from the PP1 catalytic subunit knockouts suggest that in the case of PPP1C α , even though no evidence of gene elimination in PCR was present, a decrease in the protein levels PPP1C α and foci count was observed. Similarly, a decrease in the foci count of PPP1C β was also observed whereas its PCR and western blot had negative results. PPP1C γ on the other hand failed to provide any reduction in any of the biomarkers. It is essential to keep in mind that it was a polyclonal colony of cells and no selection or isolation of pure colony of transfected cells were conducted which will impact the results of the experiments. It is hence extremely possible that half of the cells might not have been transfected and might explain the contradictory results obtained for PCR, western blot and foci count.

So far, the data appears to suggest that PPP1C α and PPP1C β might have a role to play in the pathology of DM1 as a reduction in the foci was observed. PPP1C γ however, may or may not have a role to play in DM1. Considering the isoforms of the PP1 catalytic subunits share high sequence identity (>90%), it was interesting to note that PPP1C α and PPP1C β seems to have a role to play in DM1 whereas PPP1C γ failed to produce any result. It is however important to note that even though all the isoform share a >90% sequence identity, they differ in tissue expression, and possess specific functions (46). Moreover, they associate with different regulatory subunits and as mentioned before, it is regulatory subunit that is responsible for substrate specificity rather than the catalytic subunits. Thus, the western blot data indicating that all three isoforms are expressed in KB cells is also an interesting result to have obtained.

The data therefore emphasises the fact that phosphatases are complex and therefore need to be studied in depth. All the experiments should be repeated a number of times to ensure a true conclusion is been made. Additionally, to allow the generation of more reliable data, picking up, isolating, and growing a pure colony of transfected cells might help in attaining a clear result. Further, it can be suggested that regulatory subunits can be a good target for knockouts as they bring specificity to the subunits and a combinatory knockout might set forth some interesting results.

Overall, the data support that some protein phosphatases may be involved in the Myotonic dystrophy pathway (38) however, more in depth knowledge of protein phosphatases would be required to fully understand their complex behaviour and structure and identify the ones that play a role in DM pathology.

5 OVERALL DISCUSSION

To understand the molecular pathogenesis of myotonic dystrophy and thereby some potential strategies for therapeutics, relevant compounds or therapies were tested on patient generated immortalized human KB-TeloMyoD (DM1) fibroblasts and KAGO-TeloMyoD (DM2) fibroblasts. Considering nuclear foci as the visual biomarker for the disease, the macrolide compound rapamycin, CDK12 inhibitor dinaciclib and 8081 and protein phosphatase 1 were used as potential targets to analyze any produced effects.

As mentioned above myotonic dystrophy is clinically variable and multisystemic, the action of molecular therapies could aim at several pathogenic levels of DM such as inhibition of CTG repeat transcription, degradation or inactivation of toxic mutant transcript, disruption of MBNL sequestration with the toxic RNA and regulation of CELF and modulation of Downstream Signalling pathways (22). However, compounds or therapeutics tested in this project majorly target the downstream signaling cascades for treatments.

The RNA hairpin and MBNL sequestration formed nuclear foci is the main pathogenic event of the disease and is therefore considered as a visual biomarker for all the experiments(57). Any reduction in the foci represent an effect on a key feature of the molecular pathophysiology and highlights the treatment's potential. In each experiment, certain negative or positive controls were present to ensure the reliability of the treatment.

Rapamycin, a macrolide compound known for its mTOR inhibitory action was tested in both DM1 and DM2 cells with their respective DMSO controls. Rapamycin successfully reduced the number of nuclear foci in DM1 and DM2 cells at their highest concentrations, highlighting its potential as a therapy against the disease. An EC50 value of 150uM was generated for rapamycin in both DM1 and DM2. Therefore, the data obtained suggested the possible involvement of mTOR signaling pathway in the pathology of myotonic dystrophy. Additionally, It is essential to mention that inhibitors possess the ability to affect multiple pathways and it is possible that rapamycin may act on several targets that may or may not link to mTOR(58).

Additionally, Rapamycin has been used for renal transplantation where doses as low as 1.5mg/m² to as high as 4mg/m² have been used. Moreover, in clinical trials evaluating for rapamycin in renal transplant a loading dose of 15 mg with a 5-mg maintenance dose (corresponding to 7.89 and 2.63 mg/m², respectively, based on 1.9 m² average male body area) was found to be safe(59). Furthermore, in a recent study Fktn-deficient mice with later stage dystroglycanopathy induced with daily oral dose of rapamycin for 4 weeks reduced the muscle damage and improved the muscle function. The findings of the paper suggested mTOR signalling as a potential viable therapeutic target for muscular dystrophies(59). Furthermore, findings from another paper stated IC₅₀ values of rapamycin as an immunosuppressor in the range of 0.1–300 nM(60).

Hence, the literature so far suggests the involvement of the mTOR pathway as the primary target of rapamycin. Therefore, the data obtained also compliments the data achieved in the previous literatures that state deregulation of AMPK/mTORC1 signaling, together with mild autophagy perturbation contribute to DM1 associated muscle alterations. It also stated that treatments targeting the AMPK/mTORC1 disbalance can improve muscle function(Marielle Brockhoff, 2017) (39).

Furthermore, CDK12 inhibitors dinaciclib and 8081 were tested on HSA^{LR} female and male mice whose generated gastrocnemius tissues were used to observe any changes produced in the nuclear foci due to the treatment. The data obtained suggested the possibility of the involvement of CDK12 in the DM pathology as dinaciclib produced a slight reduction in the foci clusters and a slight reduction in the foci sum intensity and foci per nucleus count though statistically it was insignificant. Previous literature by Ami Ketley, 2020 also demonstrated that a reduction in nuclear foci was attained when the DM1 cell line was treated with dinaciclib without the presence of cellular toxicity (2). Moreover, to ensure the dinaciclib treatment was effective, similar intraperitoneal injection of dinaciclib along with blind analysis was performed on an in vivo mouse model HSA^{LR} in her study. A significant reduction was observed in the number of cells containing nuclear foci and the repeat-containing transcript were also found to be significantly reduced (2). Hence, the previous literature and the promising results yielded by dinaciclib though insignificant, highlights the need for its further testing in animal DM models.

On the other hand, 8081 failed to produce any significant reduction in foci. Thus, 8081 could be tested again perhaps in DM1 cell lines and animal models to consolidate a fair conclusion. Additionally, a high background intensity was observed in majority of dinaciclib and 8081 samples which could hinder the results therefore a background subtraction step was performed in the analysis. A data calculation without background subtraction was also done to identify changes if any in the results, however no significant result was obtained from either of the dataset. The results obtained therefore could not address the aim to observe the effects of CDK12 inhibition in myotonic dystrophy and therefore highlights the need to repeat the experiment in order to gather a fair conclusion. However, the previous literature suggests that CDK12 plays a role in the regulation of transcription and processing of mRNA, and that CDK12 inhibition is expected to prevent transcription of faulty DMPK gene and thereby rescuing its toxic effects (41).

Lastly, to access the involvement and importance of phosphorylation in DM pathway, the catalytic subunits of protein phosphatase 1 were targeted for gene silencing and genetic knockout using siRNAs and guide RNA/CRISPR Cas9 respectively. Hence, PPP1C α , PPP1C β and PPP1C γ were targeted for gene editing and tested via PCR, western blot and FISH to examine its outcome on the foci and DNA mutation.

Hence, the data obtained from the PP1 catalytic subunit knockouts suggested that in the case of PPP1C α , even though no evidence of gene elimination in PCR was present, a decrease in the protein levels PPP1C α and foci count was observed. Similarly, a decrease in the foci count of PPP1C β was also observed whereas its PCR and western blot had negative results. PPP1C γ on the other hand failed to provide any reduction in any of the biomarkers. It appears that the contradictory results obtained from PCR and western blot in case of PPP1C α and PPP1C β maybe because of a short undetectable knockout in PCR or one copy of the gene was completely removed and the PCR primers fail to detect that copy, such that the band arises from a single intact copy. Thus, sequencing of the PCR bands can also be conducted to observe any single base pair change that might have not been detected in the PCR. A Synthego software for sequencing can hence be used to detect the percentage of the molecules that have been modified. It therefore confirms the need for a repetition of the experiment to reach any conclusion.

Considering the isoforms share a >90% sequence similarity (46), the data produced some interesting results. It thus appears that protein phosphatases may be involved in the pathology of myotonic dystrophy, however, more in depth knowledge of protein phosphatases would be required to fully identify the ones that play a role in DM pathology.

Another essential thing to be considered is that the results obtained were a result of polyclonal colony of cells. A polyclonal colony is a group of cells derived from multiple different parent cells, which emphasizes that no isolation of knockout cells was performed. The population of cells would therefore exhibit genetic and phenotypic diversity. This diversity can therefore manifest differences in gene expression, protein production and other cellular characteristics. The polyclonal colony of cells would show heterogeneity in terms of varying responses towards stimuli and the variations in the properties, highlighting the extreme possibility that half of the cells might not have been transfected. The contradictory results obtained for PCR, western blot and foci count could therefore be because of the same. While polyclonal cell population can be advantageous for certain research applications, it can also pose a challenge in terms of experimental control and consistency. Future experiments can hence consider growing a pure colony or a monoclonal colony of transfected cells to obtain clear results. Further, sequencing of the PCR bands can also be conducted to observe any single base pair change that might have not been detected in the PCR.

The data obtained from all the three chapters therefore addresses the aims of the project and highlights that DM pathology is way more complex than known and various signaling cascades are involved in the pathogenesis of the diseases that could be potential targets for treatments. The results thus raised some interesting conclusions which helped understand the molecular pathway of myotonic dystrophy better such as the involvement of mTOR signaling pathway in the pathogenesis of myotonic dystrophy. The results obtained also emphasized how phosphorylation can be used as a target strategy towards treatment. It suggested the possible involvement of certain phosphatases like PP1 and kinases like CDK12 in the molecular pathology of myotonic dystrophy. However, persistent repeats of all the experiments would be required to ensure the proposal of a sound conclusion.

Despite the encouraging results obtained, all the approaches showcase some limitations. The compounds or therapies tested above target the downstream signaling cascades and therefore they do not eliminate the disease-causing mutation directly or indirectly. Repeated administration of therapies would therefore be required to control the clinical symptoms of the diseases, as all the therapies only target a specific aspect of the disease, it does not cure or rescue the multisystemic disorder. Moreover, cellular uptake of certain therapies or compounds is still a challenge. Further, techniques like ASO and gene editing hold a promise for future therapy due to their ability to permanently eliminate the causing factor of the disease however, gene therapy still pose a challenge associated with specificity or selectivity and post gene editing symptoms like body's immune response or rejection (22).

6 CONCLUSION

Myotonic dystrophy is a clinically variable, neuromuscular, multi-systemic, RNA-based, autosomal dominant genetic disorder for which no cure is yet available. There are two types of myotonic dystrophy type 1 and type 2 that share a similar genetic cause and molecular pathway however are genetically quite distinct and clinically variable. The main pathogenic event involves the forming of nuclear foci in the disease and has been used as a visual biomarker in the project to identify any effects produced. There are several pathogenic levels that can be targeted in the disease, one of which is the downstream signaling cascades involved. All the therapies tested in the project are targeting different signaling pathways to understand the pathogenesis of the disease better as well as gain a lead towards producing a potential strategy to combat DM.

The aim of the project is therefore addressed by testing mTORC1 inhibitor rapamycin, CDK12 inhibitors dinaciclib and 8081 and protein phosphatase 1 on DM cell lines or mouse model to establish any effects produced on the nuclear foci or DNA mutation to understand the complex pathogenesis of the disorder better.

The key findings of the project showcase the complexity of DM pathology and the vast signaling cascades involved in the disease. The most significant data obtained in the study that also correlated with the previous literature suggested the involvement of the deregulated mTOR/AMPK pathway and Rapamycin as a potential treatment against DM. Further, involvement of CDK12 in pathology of the disease and the use of CDK12 inhibition as treatment was showcased. However, the role of CDK12 in the pathology of DM could not be significantly established, but produced some promising results highlighting the need for future study. Lastly, phosphatases, the complex and promiscuous enzymes and the phosphorylation event was used as a target to identify if phosphatases maybe involved in the DM pathology. Some interesting yet contradictory results were obtained, however, considering phosphatases are quite vast and complex, the results call for attention to further work. The results obtained also highlighted some difficulties or limitations faced during the project that helped in proposing some suggestions for future work. Overall, some interesting results were obtained that help deconvolute the DM molecular pathway and propose a potential lead for future research.

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