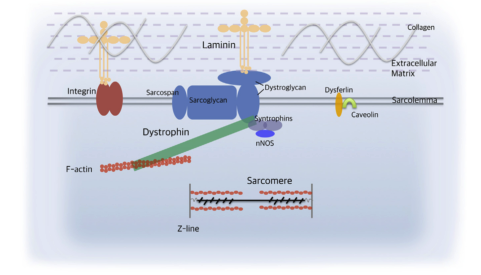
Using CRISPR/cas9 to determine the involvement of two novel NRAP variants in alpha-dystroglycan glycosylation and pathogenicity

By Ryan Duong

**Introduction**   
  Muscular Dystrophies (MD) are a group of over 30 inherited muscular disease characterized by increased skeletal muscle weakening and degeneration over time. Though there is wide variability in onset and severity across different types, all muscular dystrophies are characterized by a compromised sarcolemma and defective basement membrane in myocytes.



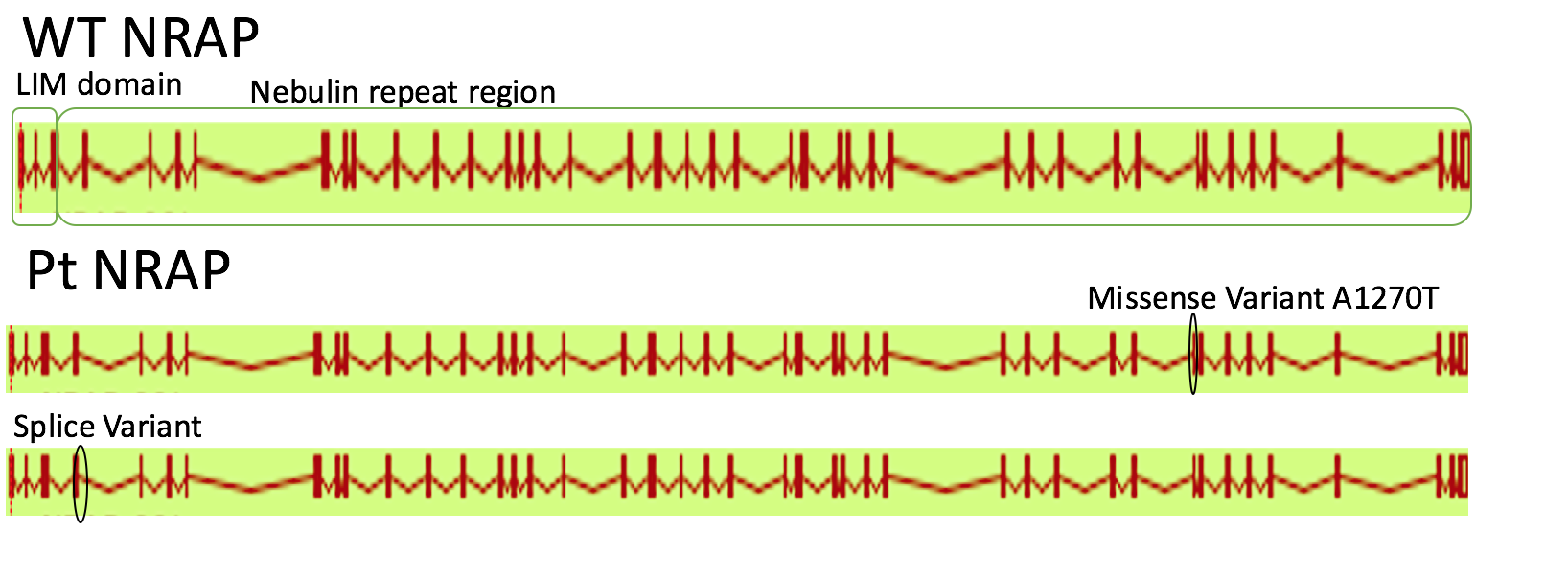
**Figure 1**: Alpha-dystroglycan in the dystrophin complex which is key in bridging the cytoskeleton and ECM in striated myocytes. Adapted from Li et al.3

Dystroglycanopathies are a common group of MD that represent a large spectrum of both neurologic and physical impairment. This group of MD is associated with reduction in of a-dystroglycan (a-dg) glycosylation.2 A-dg is a basement membrane receptor in striated muscle cells and is part of the dystrophin complex, a transmembrane protein complex responsible for anchoring the cytoskeleton to the extracellular matrix.1-3 **(Fig 1)**. A-dg functions in a diverse range of myocyte cellular processes through binding with ligands such as laminin, Agrin, and Perlecan.2,3 Binding of a-dystroglycan is dependent on its glycosylation status1, so, it is not surprising that genes associated with dystroglycanopathies encode glycosylation-related proteins2. To date, mutations in 18 genes have been associated with dystroglycanopathy diseases with varying phenotypes4-20 **(Table 1)**. However, it is estimated that only about 50% of dystroglycanopathy cases can be attributed to mutations in these 18 genes21. Remaining dystroglycanopathy-associated genes are yet to be discovered.

|  |  |
| --- | --- |
| **Disease(s)** | **Associated genes** |
| Walker-Warburg Syndrome  And Muscle Eye Brain | B3GLNT2, B4GAT1, DAG1, FKRP, FKTN, GMPPB, ISPD, LARGE, POMGNT1, POMGNT2, POMT1, POMT2, TMEM5 |
| Fukuyama Congenital Muscular Dystrophy | FKTN |
| Congenital Muscular Dystrophy with or without Cognitive Impairment | POMT1, FKRP, FKTN |
| Limb Girdle Muscular Dystrophy with or without cognitive Impairment | FKRP, POMT1, FKTN, POMT, POMGNT1, ISPD |

**Table 1:** Dystroglycanopathy diseases with associated genes4-20

Recently, a human case study presented with classic muscle eye brain (MEB) phenotype of muscular dystrophy. Immunohistochemical analysis revealed little to no glycosylated a-dg in the sarcolemma consistent with dystroglycanopathy22. Initial genetic screening for mutations in the 18 dystroglycanopathy-associated genes identified no pathogenic variants, but subsequent exome sequencing revealed two variants in NRAP protein22**(Fig 2)**. One variant was a single nucleotide polymorphism (SNP) in the first nucleotide of intron 4-5 which created a splice site mutation completely excising exon 4 from the translated protein. The other variant created the missense mutation A1270T. Parental NRAP sequencing confirmed that the father was heterozygous for the splice mutation and the mother was heterozygous for the missense mutation resulting in a doubly heterozygous splice/missense patient22**(Fig 2)**.

**Figure 2:** NRAP gene organization and representation of the doubly heterozygous splice/missense NRAP genotype (compared to wt/wt) found in a novel human case study22,24

NRAP is a large actin binding protein expressed in muscle tissues and localized to the sarcolemma24. Functionally, NRAP has been suggested to anchor terminal actin filaments in myofibrils to the membrane.25 It has also been shown that NRAP acts as an organizing center through sequential recruitment of binding partners a-actinin, filamin, and krp1 during the early steps of myofibril assembly23. Despite its close proximity to the dystrophin complex, no link between NRAP and pathogenicity has previously been established. It would be interesting to examine the causative effects between the novel doubly heterozygous NRAP genotype and dystroglycanopathy.

Model organisms are useful resource to study causative genetic agents of diseases. However, most animal models to date are complete knock-out models whereby a gene is rendered non-functional. While these knock-out models give some functional information about a gene, knock-in models mimic specific point mutations, provide a more analogous picture of human pathogenicity, and can establish a causation between a mutation and disease.

Technology in the Clustered Regulatory Interspaced Short Palindromic Repeats(CRISPR)/cas9 system offers a potential medium for the creation of knock-in mutations in an array of species from yeast to human32. The CRISPR/cas9 system is a heterologous target-specific endonuclease gene editing tool derived from bacterial adaptive immunity28**(Fig 3)**. The system comprises of a guide RNA sequence (gRNA) and cas9 protein. The gRNA binds to a specific target locus of a genome just upstream of the protospacer-adjacent motif (PAM) sequence, “NGG”. This sequence is recognized by the cas9 protein which creates a double stranded break (DSB) upstream of the PAM. To repair this DSB, the cellular DNA repair mechanism can utilize the non-homologous end joining (NHEJ) pathway whereby insertions or deletion mutations (indels) are created to ligate DNA together resulting in frameshifts and a loss-of-function knock-out. Alternatively, in the presence of exogenous donor DNA with homologous stretches on both sides of the DSB, the cell can use the homologous directed repair (HDR) strategy and fix the DSB via recombination with the homologous donor DNA. This HDR pathway is currently the pathway of choice for creating specific knock-in mutations because the exogenous DNA can be engineered to carry any mutation within its homologous sequence27,29,30.



**Figure 3:** Mechanism of CRISPR/cas9 mediated double-stranded break and repair. The cellular mechanism can create indel mutations resulting in a knock-out loss-of-function mutant via NHEJ pathway or homologously swap donor DNA which contains the target mutation via HDR.

Establishing mutant animal models of the NRAP variants via CRISPR/cas9 HDR and comparing the phenotypes to a control will show which variants are involved in the pathogenicity of MEB dystroglycanopathy as suggested in the human case study22.

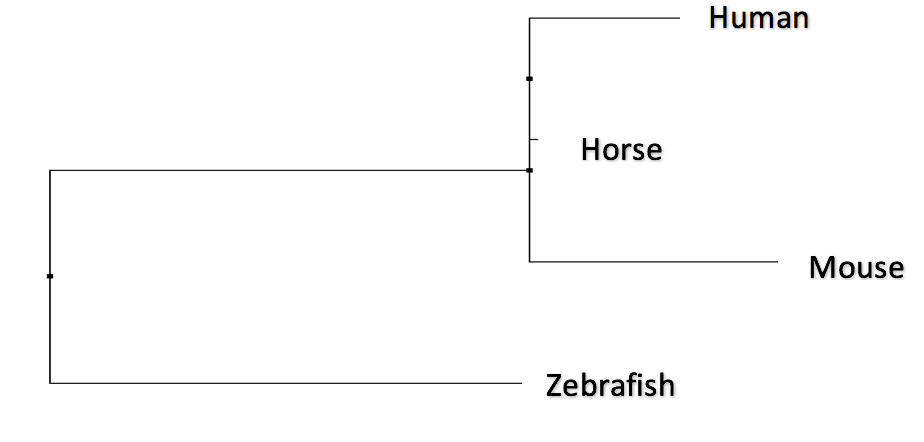
**The Experiment**

Overview

This study aims to use CRISPR/cas9 to induce specific mutations in the zebrafish NRAP protein that model the two novel variants of NRAP recently found in human.22 The following three zebrafish NRAP lines will be created: WT/splice variant, WT/missense variant, and splice variant /missense variant. Each line will be immunohistochemically stained using an antibody that recognizes glycosylated a-dg. Based on the presence of glycosylated a-dg in the sarcolemma, we can determine which if any combination of NRAP variants is responsible for the development of dystroglycanopathy.

Using zebrafish as model organisms and discovering corresponding target mutations

**A**

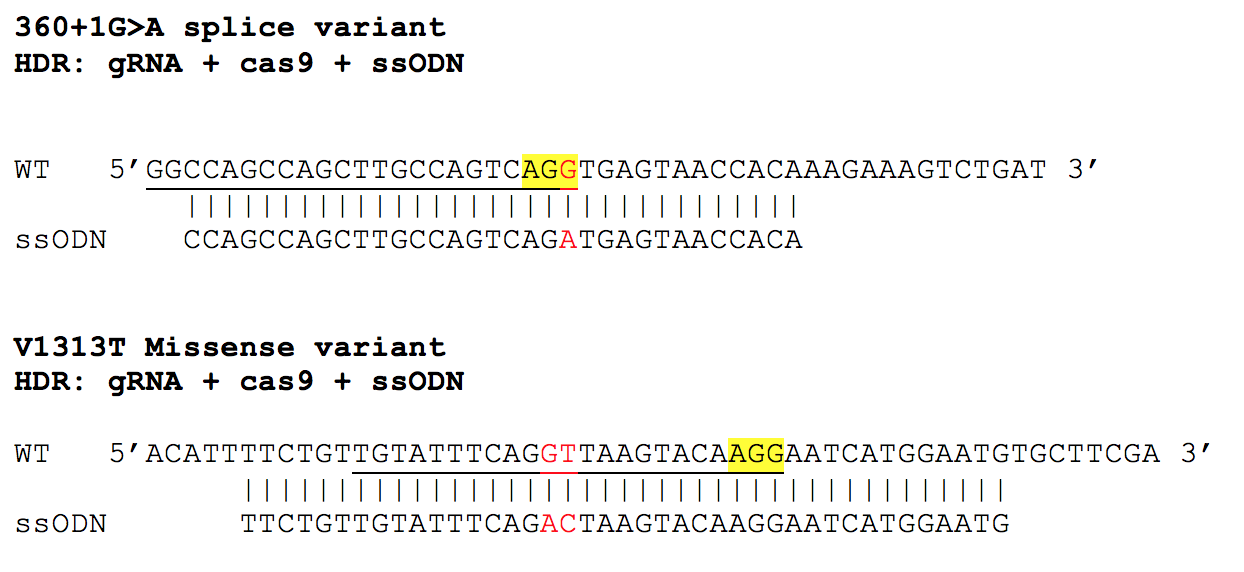
**B**



**Figure 4:** The NRAP protein is well conserved making zebrafish and appropriate model for studying its pathogenicity. A) Multiple sequence alignment of NRAP protein across multiple species33 B) Sequence alignment of zebrafish33 and human NRAP gene33 identifying corresponding target mutations in a zebrafish model

Since the 2000’s, zebrafish have been a particularly useful model for the study of MD’s. At a cellular level, myofibril structure, contractile properties, and metabolic functions are similar between zebrafish and human skeletal muscle, and many genes currently associated with MD in humans are well conserved in zebrafish3. A multiple sequence alignment of zebrafish NRAP protein shows its conservation across species and confirms the appropriate use of zebrafish as a model organism33 **(Fig 4A).** Sequence alignment of zebrafish and human NRAP genes reveal the corresponding target mutations in zebrafish NRAP needed to create the animal model33 **(Fig 4B).**

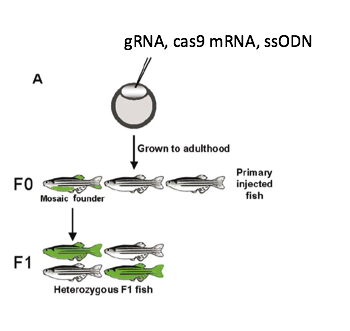
Designing CRISPR/cas9 HDR components



**Figure 5:** CRISPR/cas9 components for target HDR knock-in mutations. gRNA sequence is underlined suggesting it binds the WT strand complementary to the one shown. PAM sequence highlighted in yellow. Target mutation sites are highlighted in red. The ssODN will incorporate into the WT DNA through homologous recombination after a DSB is made upstream of the PAM.

CRISPR/cas9 remains most efficient method for creation of specific knock-in mutations27,31. Within the HDR pathway, there are multiple cases of successful knock-in generation using different forms of donor DNA including short single stranded linear DNA (6-50bp), short double stranded circular DNA, and long double stranded linear DNA (up to 4kbp)32. Armstrong et al represents the most recent and effective CRISPR/cas9 mediated knock-in generation using single stranded oligodeoxynucleotides (ssODN) of lengths 23, 32, and 100 base pairs with 2.17%, 2.12%, and 3.90% efficiency respectively29. Variables such as number of SNP target mutations and their orientation near the PAM sequence might call for differing ssODN lengths and sequences for maximum integration, but studies examining ssODN length effects and efficiency are deficient32. It is obvious the implementation of CRISPR/cas9 mediated knock-in mutation needs further refinement, but it can reasonably be hypothesized that ssODN’s centered at the DSB and with target mutation as close to the center as possible would create the highest efficiency in zebrafish given what we know about HDR recombination29,32.

CRISPR/cas9 HDR requires the injection of three parts into F0 fish at the one cell stage: gRNA, cas9 mRNA, and ssODN26 **(Fig 5).** gRNA sequences for both target mutations were obtained using an online bioinformatics database which finds a PAM sequence close to the target mutation and corresponding gRNA sequence with minimal similarity to other regions in the zebrafish genome34-35. The ssODNs were designed to be centered a few base pairs upstream of the PAM and contain homology arm lengths correlating with the distance between target mutation and predicted DSB.



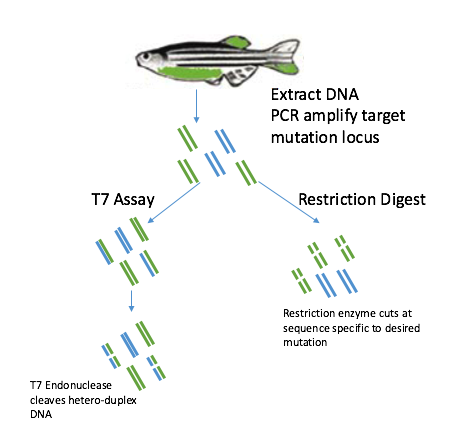
**Figure 6:** Injection and breeding strategy for the creation of heterozygous F1 zebrafish models for both NRAP variants. CRISPR/cas9 components are injected into the one-cell stage creating a mosaic founders which will be crossed with other mosaic founders with hopes that at least one contains the target mutation in the germ cells and transmits the mutation to the F1 generation. Figure adapted from Berghams et al.38

Creation of model F1 zebrafish

CRISPR/cas9 does not work fast enough in a rapidly dividing single-celled embryo creating, at best, a mosaic F0 genotype in which some genes are mutated and some aren’t30. Because of this, our overall strategy involves injecting many F0 fish, maturing them, and crossing mosaic-type F0’s with hopes that the correct mutation exists in the germ cells of at least one fish and is be passed to the F1 generation **(Fig 6).** After the creation of WT heterozygous F1 fish for both NRAP variants, those two lines will be crossed to produce the doubly heterozygous NRAP genotype. However, the frequencies of both NHEJ and HDR mediated mutations are low with NHEJ mediated knock-outs more common than HDR (about 20% versus 2%) even in presence of an ssODN 29. Thus, an early F0 screening process prior to maturing F0 generation fish can save time and money.

Using the proposed screening system **(Fig 7)**, F0 fish will first be injected at the one-cell stage with only CRISPR/cas9 NHEJ components (no ssODN) and screened for successful gRNA target specificity using a T7 endonuclease assay36,27. That is, the T7 assay will prove the proposed gRNA works. A DNA sample of F0 fish will be extracted and subjected to PCR with primers designed to amplify the target mutation locus. These PCR amplicons will be denatured and reannealed whereby some mutated amplicons will reanneal with WT amplicons creating a hetero-duplex, a WT strand base paring with a mutant strand. The amplicons will then be subjected to T7 endonuclease which is an enzyme that recognizes and cleaves only hetero-duplex DNA36. The presence of this cleavage, which can be elucidated on a gel, will indicate successful gRNA-directed cleavage.

After successful gRNA specificity is established for both mutations, the effectiveness of HDR-mediated knock in mutations with our designed ssODN’s will be tested by injecting a new F0 generation with gRNA, cas9 mRNA, and ssODN and then screening via restriction digest29. This digest calls for DNA amplicons of the target mutation locus from F0 embryos will be subjected to a specific restriction enzyme. Restriction enzymes cleave double stranded DNA at enzyme-specific recognition sequences. The restriction enzymes chosen for this screen will contain a recognition sequence that incorporates the target mutation such that a restriction enzyme will cut only amplicons in which the targeted mutation was successfully integrated. Therefore, if any DNA cleavage is seen on a gel, we can establish that our ssODN was successfully integrated into at least one cell of a F0 mosaic fish. **(Fig 8).** It is important to note that in designing primers, the WT amplicon sequence was examined to ensure it did not already contain the restriction recognition sequence prior to target mutation integration.

**Figure 7:** F0 Screening process to ensure gRNA specificity (T7) first then target mutation generation (restriction) second. 

Using this screening process, we can at least determine if our gRNA and ssODN work in general. If it does, we can justify maturing a given F0 generation and cross mosaic fish. If it doesn’t we might design different ssODN sequences or attempt a different HDR mechanism using plasmid donor DNA30-32.

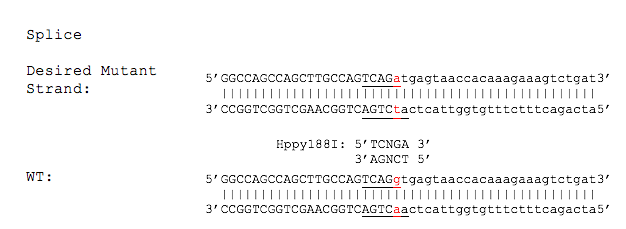
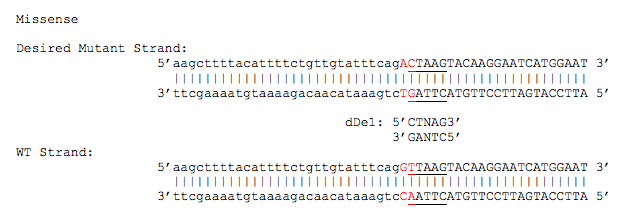
Staining of glycosylated a-dg for each NRAP variant line

The 3 zebrafish NRAP models will be immunohistochemically stained for glycosylated a-dg using the antibody IIH623. Staining will follow the same protocol as the Lin et al paper which previously demonstrated the fukutin gene’s involvement in dystroglycanopathy using a zebrafish model37.

**Discussion**

The results of this investigation will give insight into the involvement of NRAP variants in pathogenicity. If immunostaining of the zebrafish doubly heterozygous line shows decreased glycosylated a-dg in the sarcolemma similar to the fukitin mutant37, a pathogenic link between the human NRAP genotype and MEB phenotype of dystroglycanopathy23. Alternatively, if only one of the zebrafish models shows the dystroglycanopathy phenotype, only that specific variant is the cause for the patient’s disease. Finally, if staining all three models show no deficient a-dg glycosylation, then the NRAP variants are not the involved in human disease and there must exist some other undiscovered causative agent in the human.

Some potential concerns with this investigation are CRISPR/cas9 issues with low efficiency and mosaic models previously addressed via careful design of CRISPR/cas9 components and implementation of a screening system. However, another concern is that it is not certain that a g>a SNP in zebrafish intron 4-5 will result in a complete excision of exon 4 in the zebrafish NRAP protein. To date, there are no tools for comparing the splice patterns and sites of zebrafish NRAP gene with human. NRAP protein sequencing from the splice/WT model will be needed to determine if exon 4 was completely removed using this approach. If this is not the case, other HDR mechanisms might be utilized using exogenous donor DNA with extremely long homology arms of up to 2kbp that simply skips over exon 4 in its sequence though the predicted efficiency of this approach is lower.26



**Figure 8:** Specific restriction enzymes dDe1 and Hppy188I will only cut PCR amplicons from mutant cells carrying the specific targeted mutations representing the missense and splice variant respectively. No cuts will be seen in WT amplicons because the recognition sequence depends on the target mutations

The establishment of a zebrafish NRAP model will open the door for further investigation of these novel mutations and characterization of NRAP’s interactions with the dystrophin complex. In future studies, we can use these models to determine specific biochemical mechanisms of NRAP’s involvement in disease. Furthermore, successful creation of a knock-in model using CRISPR/cas9 HDR will add further insight into optimization of this developing technology.

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