Characterization and Knockdown of Zebrafish Dynamin 2

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Abstract

Dynamin 2 (DNM2) is involved in clathrin-dependent and clathrin-independent endocytosis, intracellular membrane trafficking, and the regulation of actin and microtubule networks. Mutations in *DNM2* cause both centronuclear myopathy (CNM), a congenital myopathy, and Charcot-Marie-Tooth (CMT) disease, a peripheral neuropathy. Currently, little is known about the function of *DNM2* in neuromuscular development. To address this limitation, we examined *dnm2*, a zebrafish homolog of *DNM2*. It is shown here through protein-protein BLAST analysis that the zebrafish Dnm2 is highly related to human DNM2. It is demonstrated that *dnm2* is expressed in adult zebrafish brain, spinal cord, liver, heart, and skeletal muscle tissue, which is consistent with the ubiquitously expressed human *DNM2*. Finally, morpholino-mediated knockdown of *dnm2* in zebrafish embryos results in the development of multiple developmental defects, indicating a significant role for *dnm2* in zebrafish development.

Introduction

Proper turnover of membranes and the timely transport of intracellular materials are crucial to cellular function and survival in all cells. Many of these tasks are facilitated by a family of GTPases known as dynamins[1,2]. Of distinct interest, dynamin 2 is associated with clathrin-dependent and clathrin-independent endocytosis, as well as intracellular membrane trafficking. This ubiquitously expressed protein has also been shown to participate in the regulation of actin and microtubule networks[2,3]. The human form of this gene is located on the short arm of chromosome 19, encompassing a 114-kilobase region with 22 exons[2]. Combinations of alternative splicing at two sites yield four distinct isoforms of DNM2[2]. In the rat, all four isoforms are expressed in cerebral tissue[4]. Though the exact isoform expression pattern is unknown in humans, all four isoforms are expressed in human muscle and peripheral nerve tissue[2]. The DNM2 protein consists of five distinct domains: GTPase domain, Middle domain, Pleckstrin Homology domain (PH), GTPase Effector Domain (GED), and a Proline-Rich Domain (PRD)[2,5] (See Fig. 1A).

The *DNM2* gene is an intriguing target worthy of neuroscientific examination because mutations in this gene cause two different human neuromuscular diseases (See Fig. 1A). Recently, mutations in *DNM2* have been implicated in the pathogenesis of Charcot-Marie-Tooth (CMT) disease, a genetically heterogeneous peripheral neuropathy characterized by progressive muscle weakness of the extremities[2,6,7]. *DNM2* mutations have also been linked to both familial and sporadic forms of centronuclear myopathy (CNM), a congenital myopathy[2,8]. As of yet, little is known about the function of *DNM2* in neuromuscular development and maintenance. Knockout of *DNM2* in mice results in early lethality[9], which provides little information about the

specific role of *DNM2* during development. In order to address this obstacle, this study proposes to use morpholino-mediated knockdown of *dnm2* in the piscine model *Danio rerio*.

Morpholino phosphordiamidate oligonucleotides (morpholinos; MOs) are stable uncharged water-soluble nucleic acid analogues that bind to complimentary sequences of RNA[10]. Due to the high binding affinity of MOs for RNA, these 25-subunit molecules are highly effective genetic targeting tools[11]. Morpholinos are commonly used for mRNA translation inhibition or alternative splicing of pre-mRNA[10]. Morpholinos are frequently employed to knockdown genes that function in early development and are effectively used in embryonic studies of animal models such as *Xenopus* and *Danio rerio*[10,11]. MOs are typically delivered to embryos by microinjection at the 1-4 cell stage[10]. Progeny of injected cells contain the desired oligos, and depending on the relative permeability of a species' embryonic cells, MOs may diffuse and be taken up by surrounding un-injected cells within the same embryo, a feature common to zebrafish[10].

Because mutations in the *DNM2* gene are linked to diseases affecting two essential biological systems, it is important to learn more about the specific function of *DNM2* itself. An enhanced understanding of a piscine *DNM2* will pave the way for the generation of successful models of *DNM2*- related disorders in zebrafish. This study establishes the general organization, expression, and developmental role of the zebrafish *DNM2* homolog, *dnm2*.

Methods and Materials

Danio rerio

Zebrafish were maintained in compliance with all standards set by the University of Michigan Institutional Animal Care and Use Committee (UCUCA). Wild type (AB strain) zebrafish were used in all experiments. Embryos were collected after timed mating of adult zebrafish.

Protein Homology Analysis

Human DNM2 isoform 1 and zebrafish Dnm2 amino acid sequences were compared for percent homology. Sequences were obtained from the NCBI protein database. Following the respective posted annotations, sequences were divided by domain (GTPase, Middle, PH, GED, and PRD) and compared using the Blastp algorithm through NCBI's Basic Local Alignment Search Tool (BLAST). BLAST identities are defined as the fraction of total residues which are identical. BLAST positives are the fraction of residues for which alignment scores have positive values. The most recent annotated version of the zebrafish Dnm2 amino acid sequence available through NCBI does not include the entire Proline-Rich Domain sequence, so homology analysis was performed on a comparable segment (22 amino acids) of the human PRD region.

Morpholinos

A splice-targeting morpholino (MO^{dnm2}) was designed against the donor site of exon 2 of the zebrafish *dnm2* (5'-TGCCGTGCTCATTAACACACTCACC-3'**)**. A standard control (MO^{Ctl}) was also used (5'-CCTCTTACCTCAGTTACAATTTATA-3') which is targeted against a splice-site mutant unique to human beta-globin in thalassemia[12] and has no significant biological activity in zebrafish (www.gene-tools.com). All morpholinos were constructed by Genetools.

Microinjection and Embryo Collection

Prior to injection, morpholinos were diluted with RNase-free water and 10% phenol red. MO^{dnm2} and MO^{Ctl} were injected at .3mM concentration. Constructs were injected into the yolk sack embryos at the 1-2 cell stage. Embryos were cultivated in E2 media until 24hpf. Before processing, embryos were manually dechorionated using fine forceps.

RNA Extraction and RT-PCR

Total cellular RNA was extracted from separate batches of 20 dechorionated 24hpf embryos using a QIAGEN RNeasy Mini RNA isolation kit per the manufacturer's protocol. cDNA was reverse-transcribed from 500ng total RNA using a BioRad iScript cDNA synthesis kit following the manufacturer's instructions. PCR was performed using *dnm2* or *ef1α* (control) primers:

dnm2 forward: GGCCAAAGTTGTAACCTGGA

dnm2 reverse: CGGTTTCTGCTTCAATCTCC

ef1α forward: TCACCCTGGGAGTGAAACAGC

ef1α reverse: ACTTGCAGGCGATGTGAGCAG

PCR cycles were performed as follows: 95°C for 4 min, 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s.

Total cellular RNA was extracted from adult brain, spinal cord, liver, heart, and muscle tissue isolated from 6 month-old AB fish. RT-PCR protocols listed above were utilized, and cDNA products were run on 1% agarose gels. For developmental experiments, amplified cDNA products were analyzed via gel electrophoresis and run on 1% agarose gels.

For morpholino experiments, injected 24hpf embryos were processed using the described QIAGEN and BioRad protocols and amplified using *dnm2* or *ef1α* primers. cDNA products

were run initially on 1.5% agarose gels, but later 3.0% low melting point agarose gels were used for better separation. Distinct bands were excised and cDNA was extracted using the QIAquick Gel Extraction Kit (QIAGEN) per the manufacturer's instructions. cDNA sequencing was performed by the University of Michigan DNA Sequencing Core.

Results

Protein Homology Analysis

Protein-protein BLAST analyses of the GTPase, Middle, PH, GED, and PRD domains show that human DNM2 and zebrafish Dnm2 have identities of 90%, 90%, 93%, 87 %, and 83%, and positive values of 90%, 97%, 99%, 95%, and 95% for the respective domains (See Figs. 1B-C).

DNM2 Adult Tissue Expression

RT-PCR analysis of adult zebrafish tissue reveals that *dnm2* is expressed in all five tissues. Expression was observed in liver, lung, spinal cord, brain, and skeletal muscle tissues (See Fig. 2).

DNM2 Morpholino-mediated Knock Down

An abnormal phenotype is observed in MO^{dnm2} zebrafish larvae at 24hpf (See Fig 3D). The MO^{dnm2} larvae exhibit shortened body length, increased spinal curvature, reduced blood flow, pericardial edema, and yolk sac edema.

RT-PCR and gel electrophoresis confirmed *dnm2* knockdown in the 24hpf MO^{dnm2} larvae as compared to MO^{Ctl} clutch-mates (See Fig 3A). Both MO^{dnm2} and MO^{Ctl} cDNA amplified using *ef1α* control primers exhibited bands of identical sizes. MO^{Ctl} cDNA amplified using *dnm2* primers exhibited a single band of length 247bp, whereas MO^{dnm2} cDNA produced three bands of 173bp , 247bp, and 291bp respectively (See Figs. 3A-B). Sequencing of the longest MO^{dnm2} band, 291bp in length, reveals that it consists of an unexpected splice variant in which exons 1-3 are expressed intact, but includes a short intronic segment between exons 2 and 3 (See Figs. 4A, 4D, and 5).

Discussion

DNM2 is an important target for neuroscientific investigation because mutations in the human *DNM2* gene are implicated in two neuromuscular diseases. The significance of *DNM2* is further supported by the crucial role that the DNM2 protein plays in proper cellular function. Here it is shown by protein-protein BLAST comparison that the Dnm2 homologue in *Danio rerio* is highly related to the human DNM2 isoform1 at the level of translation. The high percentage of identical amino acids in each of the protein's five domains suggests strong conservation of Dnm2 protein structure across these two vertebrate species. Furthermore, it is unlikely that either gene has experienced dramatic recombination because the observed positive percentages indicate high fidelity of amino acid sequence alignment. Such evidence validates the use of zebrafish *dnm2* in order to better understand *DNM2*.

RT-PCR was performed on brain, spinal cord, skeletal muscle, heart, and liver tissue in order to investigate the expression pattern of *dnm2* in adult zebrafish. We have shown that *dnm2* is expressed in all five tissues. This finding is consistent with expression patterns observed in other species, such as humans, in which *DNM2* is ubiquitously expressed[1]. This further supports the use of *dnm2* as a model for investigation of *DNM2*.

In order to better understand the developmental role of DNM2, morpholinos were designed to knockdown *dnm2* expression in zebrafish embryos. Morpholinos are highly effective pre-mRNA alternative splicing modifiers, making them useful tools to mediate the knockdown of gene expression[10,11]. Naturally, alternative splicing is under the direction of small nuclear ribonucleoproteins (snRNPs) which bind to introns, marking intron/exon boundaries[10]. MOs designed to target these native snRNP-binding sites can redirect splicing to produce abnormal intron-bearing mRNA, or by causing complete exon exclusion through re-directed

excision[10,11]. By blocking a native snRNP-binding site, a cryptic splice site can become activated which may complicate the interpretation of splice products due to possible partial deletions of exons or partial inclusions of introns[10]. Efficacy of splice alteration can easily be monitored by RT-PCR techniques[10,11].

We found that MO knockdown in zebrafish embryos targeting *dnm2* pre-mRNA causes an abnormal developmental phenotype. As compared to MO^{Ctl} injected embryos, MO^{dnm2} injected embryos develop shortened body lengths, increased spinal curvature, reduced blood flow, pericardial edema, and yolk sac edema. These dramatic anatomical defects suggest a significant role for *dnm2* in the early development of zebrafish. MO^{dnm2} is designed to modify alternative splicing such that exon 2 of *dnm2* is omitted and the following exons are out of frame. The expected lengths of the natural RT-PCR product and the truncated product are 247bp and 173bp. Knockdown efficiency was confirmed by RT-PCR (See Fig. 3A). Though the two expected band lengths were observed on a 1.5% agarose gel, there appeared to be an additional band of longer length.

To identify and confirm the extra band's existence, amplified cDNA was run on a 3% gel, and indeed a third band 291bp in length was easily discerned (See Fig. 3B). This third band was excised and extracted from the gel. Sequencing revealed that for this transcript, the MO^{dnm2} failed to target the exon2 splice site, but instead activated an intronic cryptic splice site downstream from exon 2 (See Figs. 4A, 4D, and 5). The included intronic sequence introduces an early stop codon, preventing the final mRNA product from proper translation (See Fig. 5). This further suggests that the observed morphant phenotype is indeed the result of *dnm2* knockdown rather than a possible dominant-negative effect carried out by a product of the unexpected third transcript.

This project has successfully shown through protein-protein BLAST analysis that the human and zebrafish homologues are highly related. We have demonstrated that morpholino mediated knockdown of *dnm2* results in a dramatic phenotype in which larvae exhibit multiple developmental deficiencies. RT-PCR analysis indicates that *dnm2* is expressed in many types of adult zebrafish tissues. Overall, zebrafish *dnm2* offers a promising model for which to learn more about human *DNM2* and its role in neuromuscular disease and development.

References

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Figures

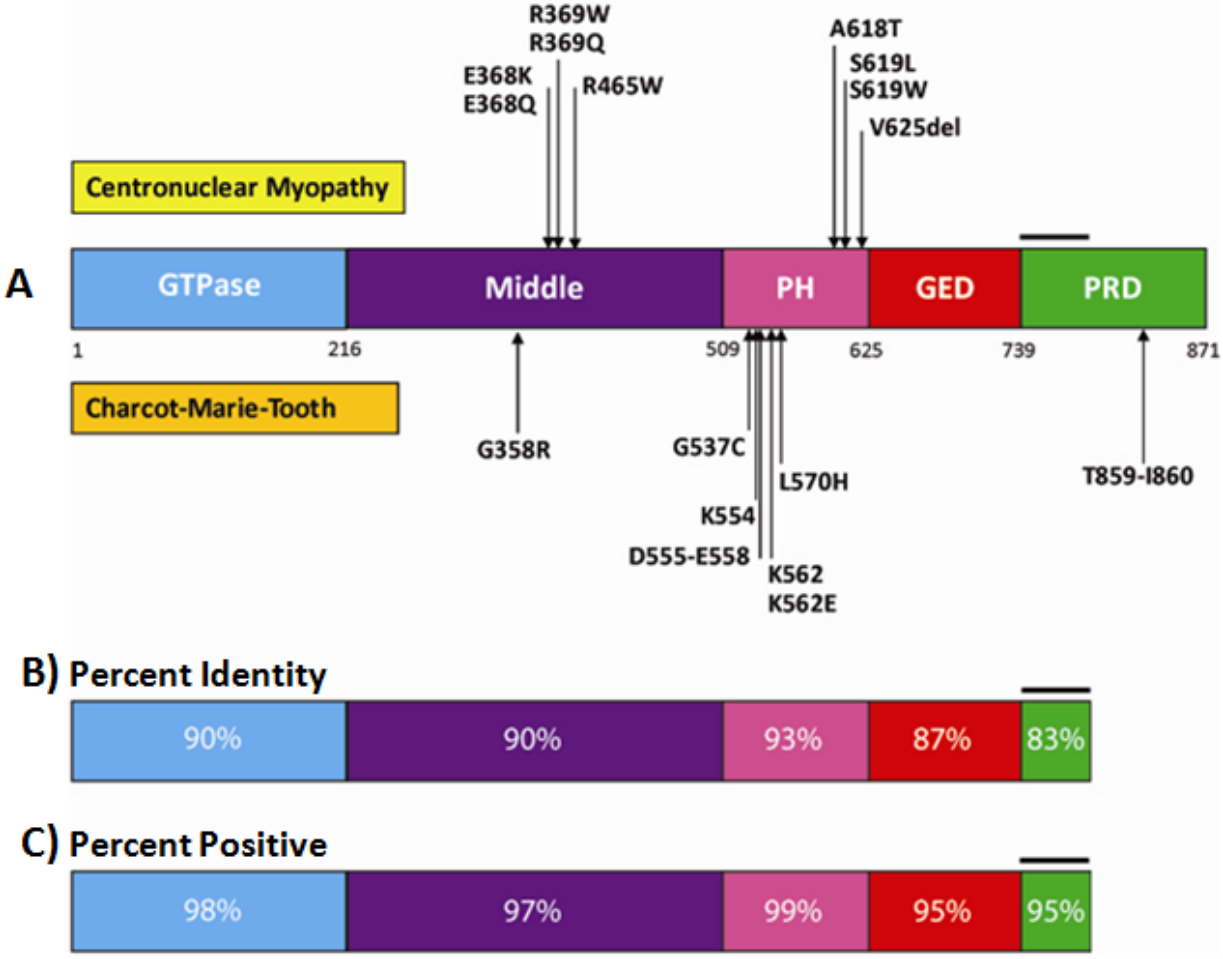

Figure 1. DNM2 mutations and homology. **A)** Schematic organization of human DNM2 showing five protein domains and positions of seventeen disease-associated mutations. Mutations above the schematic are related to centronuclear myopathy, and those below it are associated with Charcot-Marie-Tooth disease. **B-C)** Schematic organization of protein-protein BLAST comparison between human DNM2 and zebrafish Dnm2. The green labeled PRD domains in B and C are truncated as compared to that illustrated in A due to limitations in public records of the zebrafish Dnm2 PRD. The available zebrafish PRD sequence was instead compared to an equivalent length segment of the human DNM2 PRD sequence. **B)** Percentage of identical residues between respective domains. **C)** Fraction of residues for which alignment scores have positive values.

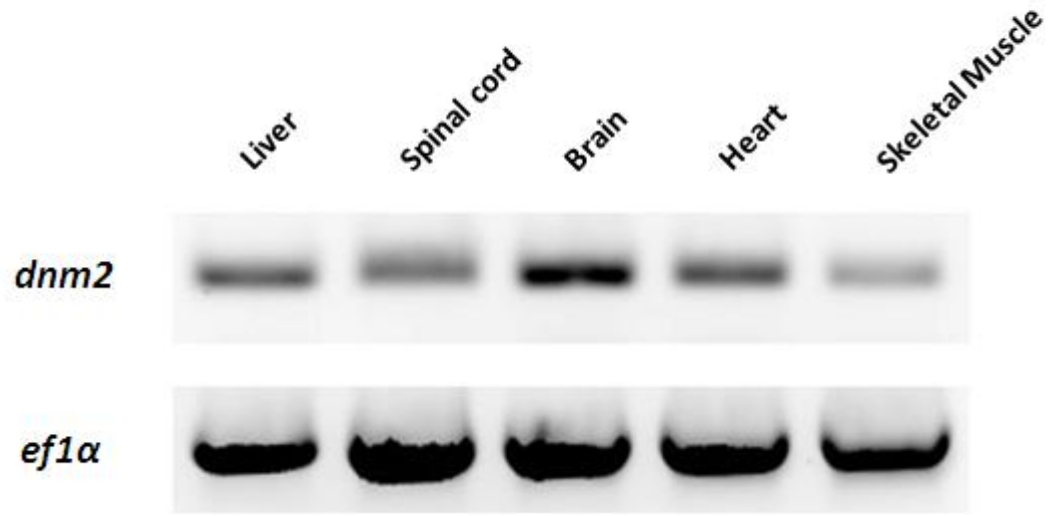

Figure 2. Expression of *dnm2* in adult zebrafish. RT-PCR was used to assay expression levels of dnm2 in tissues isolated from adult zebrafish six months of age. Primers for *ef1α* were used as an internal control. Like human *DNM2*, zebrafish *dnm2* is expressed in many tissue types.

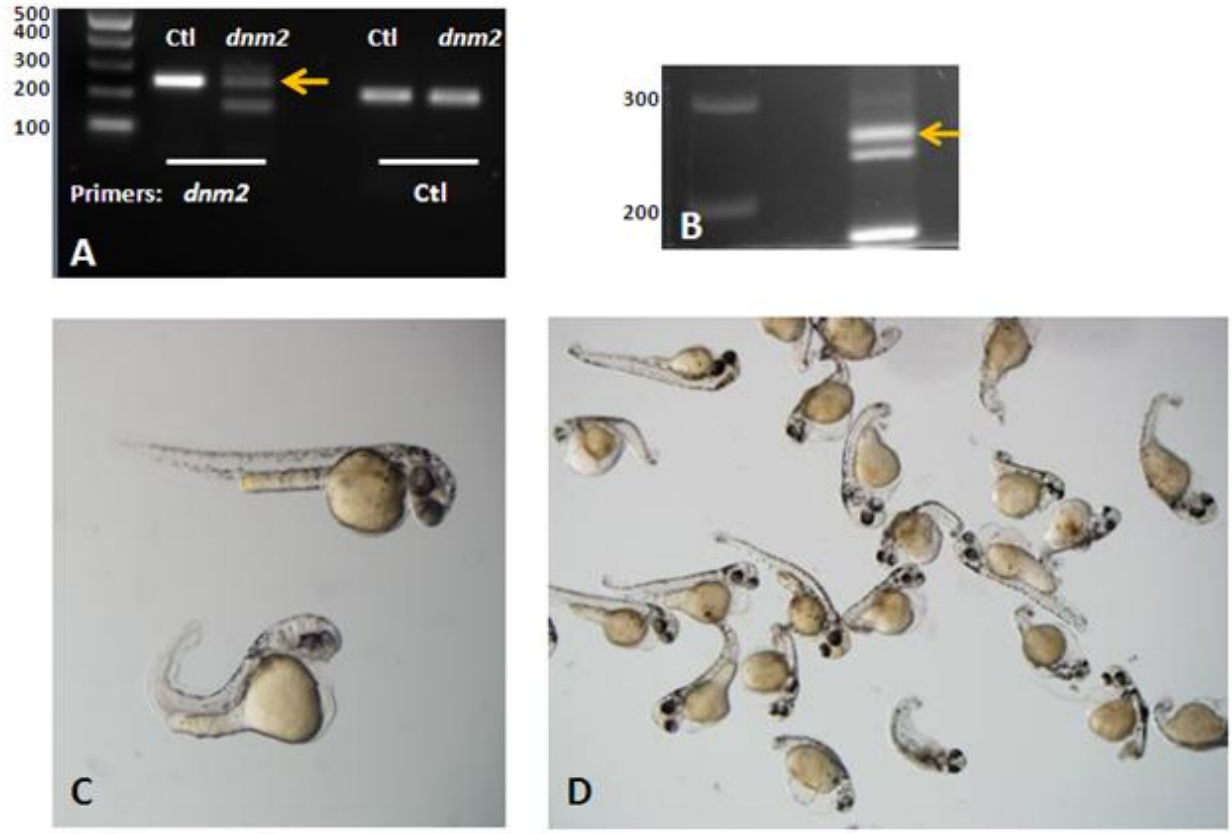

Figure 3. Morpholino-mediated knockdown of *dnm2* in zebrafish embryos. **A)** RT-PCR was used to evaluate knockdown efficiency of MO^{dnm2} in 24hpf embryos as compared to those microinjected with MO^{Ctl}. The presence of two bands of lengths 173bp and 247bp confirm *dnm2* knockdown. A possible longer third band was observed (see orange arrow). **B)** For improved resolution, cDNA from embryos injected with MO^{dnm2} was run on a higher density agarose gel. The presence of a third band of length 291bp was confirmed (see orange arrow). **C)** Side by side phenotypic comparison of a 48hpf zebrafish: wild type larva (top), and a MO^{dnm2} injected larva (bottom). **D)** Zebrafish embryos injected with MO^{dnm2} develop an abnormal phenotype observed here at 24hpf. This phenotype is characterized by shortened body length, increased spinal curvature, reduced blood flow, pericardial edema, and yolk sac edema.

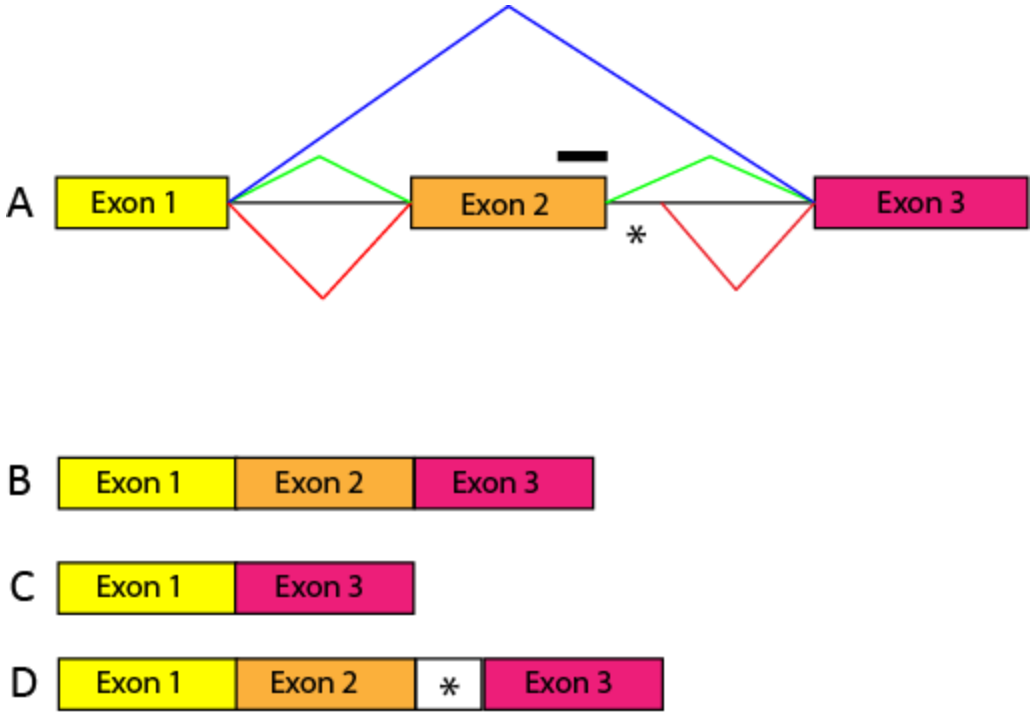

Figure 4. Schematic representation of morpholino-mediated alternative splicing of *dnm2* pre-mRNA. **A)** Illustration of splicing interactions as they relate to the first three exons of *dnm2*. Black bar above exon 2 denotes the intron/exon boundary targeted by MO^{dnm2}. The set of green lines indicate the wild type splicing mechanism (see schematic mRNA product **B**). The blue lines indicate the expected MO^{dnm2} mediated splicing mechanism in which the second exon is omitted (see mRNA schematic **C**). The set of red lines indicate the mechanism by which an activated cryptic splice site redirects alternative splicing, resulting in the addition of an intronic segment (see schematic mRNA product **D**; intronic region denoted by asterisk). **B)** wild type *dnm2* mRNA **C)** MO^{dnm2} mediated alternative splicing produces a truncated *dnm2* mRNA **D)** Alternative morpholino-mediated splice product in which an activated cryptic splice site allows the transcript to retain exons 1-3, but appends an intronic segment downstream of exon 2 (intronic region denoted by asterisk).

GGCCAAAGTTGTAACCTGGATCTGCCGCAGATAGCAGTTGTCGGAGGACAGAGCGCCGGAA
AAAGCTCCGTGCTGGAAAATTTCGTCGGCAGGGATTTCCTTCCGCGAGGCTCAGGCATCGTC
ACCCGCAGGCCTCTCATTCTGCAGCTAGTAAACAACAAAGCCGGTGAGTGTGTTAATGAGCA
CGGCATAACTGCAAAGTCTTCCTAGAATATGCAGAGTTCCTACACTGCAAAGGGAGGAAGTT
CGTGGACTTTGATGAAGTCCGGCAGGAGATTGAAGCAGAAACCG

Key:

Exon 1 Exon 2 Exon 3 Intronic Sequence Early Stop Codon

Figure 5. Sequence of the 291bp RT-PCR product includes an unexpected intronic region (denoted in black) as a result of an activated cryptic splice site. The intronic region introduces an early stop codon (denoted in red) which prevents proper translation of the transcript. This indicates that the abnormal phenotype observed in MO^{dnm2} injected embryos is a result of *dnm2* knockdown as opposed to a dominant-negative effect imposed by the alternative transcript.