Dok-7 promotes slow muscle integrity as well as neuromuscular junction formation in a zebrafish model of congenital myasthenic syndromes

Juliane S. Müller*, Catherine D. Jepson, Steven H. Laval, Kate Bushby, Volker Straub and Hanns Lochmüller

Institute of Human Genetics, International Centre for Life, Newcastle University, Central Parkway, Newcastle Upon Tyne NE1 3BZ, UK

Received December 7, 2009; Revised and Accepted February 2, 2010

The small signalling adaptor protein Dok-7 has recently been reported as an essential protein of the neuromuscular junction (NMJ). Mutations resulting in partial loss of Dok-7 activity cause a distinct limb-girdle subtype of the inherited NMJ disorder congenital myasthenic syndromes (CMSs), whereas complete loss of Dok-7 results in a lethal phenotype in both mice and humans. Here we describe the zebrafish orthologue of Dok-7 and study its in vivo function. Dok-7 deficiency leads to motility defects in zebrafish embryos and larvae. The relative importance of Dok-7 at different stages of NMJ development varies; it is crucial for the earliest step, the formation of acetylcholine receptor (AChR) clusters in the middle of the muscle fibre prior to motor neuron contact. At later stages, presence of Dok-7 is not absolutely essential, as focal and non-focal synapses do form when Dok-7 expression is downregulated. These contacts however are smaller than in the wild-type zebrafish, reminiscent of the neuromuscular endplate pathology seen in patients with DOK7 mutations. Intriguingly, we also observed changes in slow muscle fibre arrangement; previously, Dok-7 has not been linked to functions other than postsynaptic AChR clustering. Our results suggest an additional role of Dok-7 in muscle. This role seems to be independent of the muscle-specific tyrosine kinase MuSK, the known binding partner of Dok-7 at the NMJ. Our findings in the zebrafish model contribute to a better understanding of the signalling pathways at the NMJ and the pathomechanisms of DOK7 CMSs.

INTRODUCTION

Dok-7 (‘downstream-of-kinase 7’ or ‘docking protein 7’) represents the most recent addition to the Dok family of small cytoplasmic signalling adaptor proteins (1). Dok-7, like all other members of the Dok family, contains a pleckstrin homology (PH) and a phosphotyrosine binding (PTB) domain in its N-terminal half, as well as Src homology 2 (SH2) binding motifs in the C-terminal region. Dok-7 is expressed in heart and at neuromuscular junctions (NMJ) in skeletal muscle, where it activates the muscle-specific receptor tyrosine kinase MuSK (1). The signalling pathway involving Dok-7, MuSK, rapsyn and nerve-derived agrin is crucial for the process of clustering high numbers of acetylcholine receptors (AChRs) at the postsynaptic muscle membrane during NMJ formation. As with mice lacking MuSK, agrin or rapsyn (2–4), Dok-7-deficient mice were immobile and died shortly after birth (1). No AChR clusters were detected in the endplate area of the diaphragm muscle in Dok-7−/− mouse embryos indicating that Dok-7 is indeed an essential protein for NMJ formation (1).

We and others subsequently identified autosomal recessive mutations in the human DOK7 gene that underlie a distinct type of congenital myasthenic syndrome (CMS) associated with a weakness predominantly of the limb-girdle muscles (5–9). This specific form of CMS is characterized at the structural level in muscle by abnormally small and simplified NMJ or degenerating junctional folds, but normal AChR density in relation to junctional fold size (8,10). DOK7-related CMS constitutes a major CMS subgroup which may present with a severe clinical phenotype and patients do not respond well to the conventional CMS treatment of acetylcholinesterase inhibitors.

*To whom correspondence should be addressed. Tel: +44 1912418668; Fax: +44 1912418770; Email: juliane.mueller@ncl.ac.uk

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Figure 1. The zebrafish Dok-7 protein: sequence analysis. (A) Predicted exon/intron structure of the two zebrafish Dok-7 isoforms. The shorter transcript is composed of seven exons, the longer transcript of 11 exons. Green boxes symbolize common exons shared by both transcripts, diagonally striped pattern symbolizes sequences unique for one of the transcripts. MO1, MO2: binding sites of the antisense morpholino oligonucleotides used to knock down zDok-7 expression; MO1 is a translation blocking morpholino binding to the region surrounding the start codon. MO2 is a splice morpholino binding to the splice donor site of exon 2. Both morpholinos are targeted to knock down both zDok-7 transcripts. (B) Alignment of protein sequences of human, mouse, puffer fish (Fugu) Dok-7 and the predicted zebrafish Dok-7 protein sequence. We used the translated short zDok-7 transcript for the alignment. Amino acids identical in all four proteins are coloured in red, similar amino acids in purple. Sequence alignment was done using ClustalW2 program: http://www.ebi.ac.uk/Tools/clustalw2/index.html. Sequences used for alignment: human Dok-7 accession number: AB220918, mouse Dok-7 accession number AB220919 and puffer fish Dok-7 accession number: AB220920.

A homozygous DOK7 splice site mutation, c.331 + 1G>T, was identified in a consanguineous family with three children affected with lethal FADS (fetal akinesia deformation sequence) (11). Although no RNA analysis was performed, the nature of this mutation suggests that virtually no functional Dok-7 is expressed in these patients and implies that complete loss of Dok-7 is also lethal in humans.

Although Dok-7 mutants and protein domains have been extensively studied in cultured myotubes (8,12,13), crucial questions regarding their pathogenic mechanisms remain open: for example, the in vivo role of Dok-7 at the innervated endplate region is still not well understood. Considering the effect of DOK7 mutations on NMJ structure rather than AChR density, it seems likely that Dok-7 function extends beyond amplifying AChR clustering and that in vivo Dok-7 has a more complex role during NMJ development and maturation. To address this, we investigated the in vivo function of Dok-7 in zebrafish. The development of the fish embryo occurs externally and is not dependent on a functional heart or lungs, and so is not subject to the problem of neonatal lethality as observed in Dok-7 knockout mice. Previous studies of Dok-7 function indicate that Dok-7 is involved in the very early stages of NMJ formation during embryogenesis. The transparent embryo and external embryonic development of zebrafish greatly facilitates in vivo imaging at these early stages in comparison with mouse embryos. Although overall muscle structure of zebrafish differs from mammalian muscles (14), NMJ formation occurs in a similar stepwise sequence in zebrafish as it does in mammals. In the first step, before the axon of the motor neuron reaches the muscle fibre, a band of AChR forms in the central region of the muscle (prepatterned AChR clusters) (15–18). The second step of NMJ formation occurs upon arrival of the axonal growth cone when contact between nerve and muscle is established in the first neuromuscular synapses (15,16). Shape, number and distribution of these synapses change later on in zebrafish when motor axons branch out and AChR clusters cover the entire myotomal surface (16).

Here, we characterize the zebrafish orthologue of Dok-7 and show that it is involved in all stages of NMJ formation. Deficiency of Dok-7 leads to impaired motility and a decrease in NMJ size in zebrafish embryos and larvae, consistent with the observations previously made in patients with DOK7 mutations. Furthermore, we provide evidence that, in addition to its function at the NMJ, zebrafish Dok-7 might be involved in maintaining muscle fibre structure. This finding uncovers a previously unknown role for Dok-7 and might increase our understanding of the unusual clinical phenotype of DOK7 patients and their unexpected lack of responsiveness to esterase inhibitors.

RESULTS

The zebrafish Dok-7 gene

A BLAST search of zebrafish transcripts with the human Dok-7 protein sequence as input revealed the presence of a potentially homologous zebrafish protein (hypothetical gene LOC557881, mRNA sequence XM_681035); the gene encoding this protein is located on zebrafish chromosome 1. Comparison of the predicted zebrafish Dok-7 protein with its human counterpart showed significant similarity in the protein sequence, protein domain organization and exon/intron structure on the genomic level (Fig. 1). Moreover, syntenic relationship to flanking genes (e.g. LRPAPI, RGS12) on human chromosome 4p16 is maintained as well, reinforcing the evidence that LOC557881 might indeed represent the zebrafish Dok-7 orthologue. No other similar protein was found in the new zebrafish genomic sequence assembly Zv8, either with the human Dok-7 or the zebrafish LOC557881 sequence as BLAST input. Only one Dok-7 gene had previously been
Figure 1. Continued.
identified in the completely sequenced puffer fish (*Takifugu rubripes*) genome (1) further indicating that LOC557881 (termed zDok-7 in the following) is the only zebrafish Dok-7 orthologue.

The zDok-7 gene in the database is predicted to be composed of 11 exons, whereas the known human Dok-7 gene has seven exons. The high degree of overall similarity of exons 1–7 of the two proteins and a human transcript database entry (accession number AK075037) that is very similar to zDok-7 exons 7–11 prompted us to closely investigate Dok-7 transcripts in both zebrafish and humans. We were able to amplify human Dok-7 transcripts that contained additional 3' exons 8–10 from cDNA obtained from primary human muscle cultures (data not shown) and we also identified a zebrafish transcript with a termination codon in exon 7 after eight amino acids specific for this shorter transcript. These findings indicate that there might be two Dok-7 isoforms expressed in both human and zebrafish: a short isoform containing seven exons and a long isoform with 11 exons in zebrafish and 10 exons in human (Fig. 1A).

The short zebrafish Dok-7 transcript is predicted to give rise to a protein of 537 amino acids (published human Dok-7: 504 amino acids), the longer zebrafish transcript encodes a protein of 664 amino acids. Almost 50% of the amino acids of the shorter zDok-7 protein are identical to the human Dok-7 sequence; the degree of homology between the two proteins is especially high within the first 250 amino acids composing the PH- and the PTB domains (Fig. 1B).

**Analysis of zDok-7 expression**

In mammals, Dok-7 is expressed in skeletal muscle and in heart; protein localization in skeletal muscle is restricted to the NMJ (1). In zebrafish, semi-quantitative RT–PCR analysis of zDok-7 expression at different time points of development showed that zDok-7 transcripts can first be detected around 10 hpf, coinciding with the time when somite segmentation begins. Primers selected for RT–PCR analysis amplify both short and long zDok-7 isoforms at the same time. No Dok-7 transcripts are present during the blastula and gastrula developmental stages. The highest levels of Dok-7 transcripts are detected between 19 and 24 hpf, corresponding to the time of the formation of the first neuromuscular synapses. zDok-7 expression is maintained throughout embryonic and larval development until adulthood. The expression pattern of zDok-7 is similar to the expression of AChRα1 subunit, an NMJ protein (Fig. 2A).

To investigate spatial expression of zDok-7 transcripts, we performed *in situ* hybridization. zDok-7 expression was detected in caudal, developing somites until approximately 24 hpf when somite segmentation is completed (Fig. 2B). Once the formation of a somite is finalized, zDok-7 transcripts disappear (Fig. 2B, rostral segments). At later time points (48 hpf and older), we could not detect zDok-7 expression by *in situ* hybridization, although we still obtained RT–PCR amplification products. This might be due to a lower level of zDok-7 expression or a more dispersed distribution of transcripts within the somites.

**Knockdown of zDok-7**

Dok-7 has been shown to be localized to NMJs in mice and humans. Similarly, in zebrafish Dok-7 is expressed at the appropriate time to be involved in NMJ formation. In order to examine whether Dok-7 plays a role in NMJ formation in zebrafish, we decided to downregulate its expression and to study NMJ structure and formation in the absence of zDok-7.

We designed two antisense morpholino oligonucleotides to knock down zDok-7 expression: MO1 directed against the start codon thereby blocking translation and MO2 binding the splice donor site of exon 2 (see Fig. 1A). Both MOs target both the short and long isoform of zDok-7, but the advantage of MO2 lies in the fact that knockdown efficiency of the knockdown can be monitored via RT–PCR. MO2 leads to
skipping of exon 2, frameshift and a premature stop codon in exon 3 after 25 missense amino acids. Both morpholinos were independently injected into one to four cell stage embryos which were then analysed up to 120 hpf. Both zDok-7 MOs produced identical phenotypes. Data shown in the following are all from experiments performed with MO2.

Muscle development in the zebrafish embryo starts at 10 hpf with the segmentation of the first somite. In the following hours, one somite forms every 30 min; somite formation occurs in a rostral-to-caudal temporal sequence with the somites located at the very tip of the tail forming last (19). By 24 hpf somite segmentation is complete. NMJ formation starts at 16 hpf with the outgrowth of primary motor neuron axons from the spinal cord in the most rostral segments (20,21). In common with muscle development, NMJs develop in a rostral-to-caudal sequence.

zDok-7 morphants display no obvious morphological abnormalities at 24 and 48 hpf except for a slightly shorter tail (Fig. 3). Approximately 10–15% of MO-injected embryos have abnormally shaped yolk sacs, cardiac oedema and a slight upward curl of the tail at 48 hpf. It is unclear whether these morphological changes are specific, as it has been previously described that pericardial oedema might be a non-specific effect of MO injections. The slightly shorter body becomes more apparent at 5 dpf; at this time point, more severely affected larvae have a curled tail and cardiac oedema.

Knockdown of NMJ proteins usually affects motility and swimming behaviour of injected embryos and larvae. Development of locomotor behaviour in zebrafish is composed of a defined sequence of movement abilities. The first locomotor behaviour stage observed is spontaneous, slow, alternating tail movements inside the chorion beginning at 17 hpf (22). When monitored at 24 hpf, zDok-7 MO-injected embryos are able to perform these tail twisting movements in their chorion but at lower frequency. While non-injected embryos coil in their chorion on average 3.69 times in 10 s, zDok-7 MO-injected embryos perform on average only 1.48 coils in the same time (standard control MO-injected embryos: 4.38 coils per 10 s, 15 embryos were observed for each category) (Supplementary Material, Videos).

Zebrafish embryos hatch from their chorion between 48 and 60 hpf; once the embryos have left the chorion they respond to a touch stimulus by rapidly swimming away from the stimulus source. Most zDok-7 MO-injected embryos display delayed hatching or are not able to leave their chorion unaided. When freed from their chorions at 48 hpf around half of zDok-7 MO-injected larvae display impaired swimming and abnormal touch-evoked response. The abnormal motility ranges from slow swimming, circling movements, a twitch or no movement at all as response to a touch stimulus (see Supplementary Material, Videos). These swimming defects seen in injected embryos persist at least until 5 dpf.

zDok-7 is required for AChR prepatterning but not for focal innervation

Considering the motility defects of zDok-7-deficient embryos and larvae, and the previously known function of Dok-7 in mammalian cells and the mouse model, we set out to examine muscle and NMJ structure in the zDok-7 MO-injected embryos and larvae.

NMJ formation occurs in well defined stages in zebrafish. The first step is the appearance of diffuse AChR clusters in a central band along the middle of the myotome well before the motor axon growth cone reaches the muscle (16). This process is known as prepatterning and it is also present in mammalian muscle (18). Prepatterned Dok-7 clusters were absent in Dok-7 knockout mouse diaphragms (1).

To investigate NMJs in zebrafish we assayed AChR localization by labelling the receptor with fluorescent bungarotoxin and stained motor axons with synaptic vesicle protein 2 (SV2) antibody. As zebrafish muscle development proceeds from rostral to caudal, rostral segments have been contacted by motor axons at 24 hpf, whereas in the most caudal segments the axonal growth cone has not extended yet and the myotomes still display prepatterned AChR clusters. In contrast to non-injected controls, in zebrafish embryos injected with anti-zDok-7 MO, AChR prepatterning is absent or very reduced (see Fig. 4A) in caudal segments at 1 dpf.
Prepatterned AChR clusters form on the adaxial muscle cell layer adjacent to the notochord in advance of the extending motor axon. Most adaxial muscle precursor cells undergo a radial migration to the surface of the myotome, where they form a layer of slow-twitch muscle cells. A small number of adaxial cells, however, remain in the proximity of the notochord; these cells are the so-called muscle ‘pioneers’ (14). The space between the newly formed slow muscle cell layer and the notochord is invaded by fast-twitch muscle cells (14). At the stage where the axon reaches the muscle, most

Figure 4. Neuromuscular junctions in absence of Dok-7. Non-injected control embryos (top panels) and zDok-7 MO-injected embryos (bottom panels) were stained for postsynaptic AChR (α-bungarotoxin, red staining) and presynaptic nerve endings (SV2 antibody, green staining). Scale bars: 50 μm. (A) AChR clusters at 24 hpf in caudal somites. In non-injected embryos, prepatterned AChR clusters are arranged in a central zone on the myotome in segments that have not yet been reached by motor axon growth cones (white box). No or very few scattered prepatterned clusters are observed in embryos injected with zDok-7 MO. (B) Neuromuscular junction formation at 24 hpf in rostral somites. Focal synaptic contacts can be detected in both non-injected and MO-injected somites (arrows), in the injected embryos the AChR clusters seem smaller. (C) Neuromuscular junctions at 48 hpf in non-injected control embryos (top row), zDok-7 MO-injected embryos with normal swimming behaviour (movers, middle row) and zDok-7 MO-injected embryos with impaired motility (non-movers, bottom row). Distributed and myoseptal AChR clusters are present in zDok-7-deficient embryos but overall size and number of distributed clusters is reduced, especially in embryos with impaired swimming. (D) Neuromuscular junctions at 120 hpf in non-injected control embryos (top row), zDok-7 MO-injected embryos with normal swimming behaviour (movers, middle row) and zDok-7 MO-injected embryos with impaired motility (non-movers, bottom row). Branching patterns of distributed AChR clusters are less elaborate in MO-injected embryos making AChR staining on the myotomal surface appear more punctate, whereas myoseptal AChR localization is slightly increased.
Figure 4. Continued.
adaxial cells except muscle pioneers have migrated laterally (15,23). The axonal growth cone forms a first contact with the postsynaptic side on the surface of muscle pioneers. At this site, some of the prepatterned aneural AChRs are incorporated there into neural clusters (15,23). Clusters not contacted by growth cones will disappear. In embryos injected with zDok-7 MO, there are no prepatterned AChR clusters on adaxial cells or on muscle pioneer cells. Once the axonal growth cone reaches the muscle pioneers, however, AChR clusters appear apposed to the axon. A colocalization of SV2 and bungarotoxin staining can be seen in these spots (Fig. 4B), although size and staining intensity of the AChR clusters is reduced in comparison with non-injected embryos. These contacts on the horizontal midline are very likely to represent the focal innervation points in the zDok-7 morphants.

Motor axon growth cones subsequently contact fast muscle fibres and form neural ‘en passant’ synapses there. These clusters on the fast fibres are smaller than the ones on the muscle pioneers [(15), and Fig. 4B, non-injected embryo]. The focal synapses on fast fibres are correctly formed in zDok-7 MO-injected embryos (Fig. 4B) indicating that zDok-7 might not be essential for this process.

**Reduction of zDok-7 expression results in less distributed innervation**

After the formation of a first equatorial line of focal innervation contacts in the centre of each myotome distributed AChR clusters on the entire myotomal surface and of myoseptal clusters form (24). In zDok-7 MO-injected embryos, we observed reduced AChR cluster size and density of distributed synapses at 48 hpf. At the same time, there is a slight increase of AChRs localized at myosepta (Fig. 4C). In order to measure the reduction of AChR cluster number and size, we quantified the total α-bungarotoxin positive area in the somites of MO-injected embryos at 48 hpf and compared it with non-injected controls. The total area occupied by AChRs was significantly smaller in Dok-7-deficient embryos (0.58%, compared with 1.04% in non-injected controls, P = 0.005).

The aberrant AChR staining pattern persists at 5 dpf: at this time point an increased AChR density at myotomal borders can be observed, alongside smaller AChR clusters and less elaborate branching on the rest of the myotome, resulting in a punctate AChR staining (Fig. 4D). Embryos and larvae injected with standard control morpholino have a normal NMJ staining pattern at 48 and 120 hpf (Supplementary Material, Fig. S1).

The reduction in distributed cluster number and size in zDok-7 morphants at 48 hpf ranges from just slightly to very reduced. We decided to test whether this variability of an NMJ phenotype is correlated to swimming abilities of zDok-7 morphants. To investigate this, we manually dechorionated zDok-7-injected embryos at 48 hpf and studied their touch-evoked escape response. Morphants with normal motility (movers) were separated from morphants with impaired touch response (non-movers). Staining of AChRs and motor neurons in the two groups revealed that there is indeed a correlation between the amount of AChR clusters present in the muscles and motility: All non-movers have clearly reduced AChR cluster numbers/size whereas movers have only slightly reduced or even normal AChR staining patterns at 48 hpf. At 5 dpf, the difference between morphants with normal and impaired motility becomes even more apparent, as the latter have often a curved tail or pericardial oedema. NMJ staining in zDok-7 morphants however differs from non-injected controls regardless of swimming abilities of examined larvae; the majority of morphants with normal swimming do still display an abnormal AChR staining pattern.

**Analysis of muscle fibre structure in zDok-7 morphants**

The finding that zDok-7 expression is highest in not yet fully differentiated somites prompted us to examine whether a loss of zDok-7 also influences muscle fibre development or structure. We investigated muscle fibre structure by staining fast-twitch muscle fibres with the F310 antibody (stains fast-twitch myosin) and slow-twitch muscles with the F59 antibody (stains slow-twitch myosin). Lateral migration of adaxial cells was not impaired in zDok-7 morphants at 24 hpf and the slow muscle fibre staining looked normal at this time point. At 48 hpf, however, F59 staining showed abnormal alignment of fibres with gaps between fibres in MO-injected embryos with motility defects (Fig. 5); injected embryos with normal motility had normal slow muscle fibres. No abnormalities were seen in fast muscles with the F310 staining in zDok-7 MO-injected embryos (Supplementary Material, Fig. S2). The ‘bowed’ slow-muscle fibres seen in morphants with impaired swimming are also present at 5 dpf (Fig. 5C). A costaining with the F59 antibody and phalloidin (which stains actin filaments in all muscle fibres) indicates that the gaps between the slow fibres might be filled by protruding fast fibres from adjacent muscle layers (Fig. 5B).

As it has been reported previously that a complete lack of AChR expression leads to a wavy staining pattern of slow muscle fibres (25), we examined whether the slow muscle fibre arrangement defect seen in zDok-7 morphants is related to reduced amounts of AChR clustering, and would therefore be associated with reduction in other NMJ proteins. We chose to compare zDok-7 morphants with morphants with a similar NMJ phenotype. From the range of published zebrafish NMJ mutants, ennui and unplugged mutants have the most similar phenotype to zDok-7 morphants at 48–120 hpf with reduced but not completely absent AChR clustering and slow swimming escape response (26,27). As the gene responsible for the ennui mutant has not yet been determined, we decided to compare zDok-7 morphants with unplugged morphants, caused by mutation in the zebrafish homologue of the muscle-specific kinase, MuSK (zMuSK).

We used a morpholino targeting only the unplugged full-length isoform, as the SV1 isoform has axon pathfinding roles that might possibly interfere with muscle development (23,27). Microinjection of the unpluggedFL MO reproduced the previously published morphant phenotype (23): prepatterned AChR clusters form on all myotomes but fail to develop into synaptic contacts with motor neurons at 24 hpf. By 48 hpf no or very few small distributed AChR clusters are present, whereas myoseptal AChR cluster density is increased (Fig. 6). At 5 dpf recovery is observed with the appearance of very small distributed AChR clusters on the entire myotome surface; at this time point, unpluggedFL mor-
phant and zDok-7 morphant AChR staining patterns are almost identical (27). In contrast to zDok-7 MO-injected embryos, slow-twitch muscle fibre staining in unplugged FL MO-injected embryos at 48 hpf is normal (Fig. 6), although the latter have a more severe NMJ phenotype at 48 hpf and also impaired motility. This indicates the disruption of the pattern of slow muscle fibres observed in zDok-7-deficient embryos, the muscle fibres have a bowed appearance with gaps between fibres (arrow), in particular in embryos with motility defects.

**DISCUSSION**

Here, we describe the zebrafish orthologue of Dok-7 and we use knockdown of protein expression via antisense morpholinos to investigate its function in zebrafish muscle. We show that the fish model recapitulates some of the NMJ pathology of DOK7-related CMS and can offer novel insights into disease mechanisms and the process of NMJ development.

In mouse and humans, Dok-7 is expressed in skeletal muscle and is concentrated at postsynaptic sites of the NMJ, colocalizing with AChR clusters. Dok-7 has been shown to interact with the muscle-specific receptor kinase MuSK in cultured C2 mouse myotubes (1,12). In zebrafish, we identified a single protein homologous to mammalian Dok-7. As in mammals, Dok-7 transcripts are detected in muscle in zebrafish, more precisely in young developing somites of the tail during the first day of embryonic development. Expression is either reduced or is less confined to one area once somite development is finalized. This expression pattern is reminiscent of the pattern seen for the zebrafish MuSK orthologue unplugged described by Zhang et al. (27) and suggests zDok-7 may be expressed by premigratory adaxial muscle precursors. These adaxial cells are located next to the notochord at the start of muscle development and migrate laterally later (14). The first AChR clusters and
synaptic contacts between motor neurons and muscle are found in these adaxial cells and muscle pioneers. The strong expression of zDok-7 transcripts in adaxial muscle precursors before 24 hpf indicates that zDok-7 is expressed at the right time and in the right place to interact with MuSK/ unplugged during NMJ formation.

zDok-7 at different stages of NMJ development

We examined Dok-7 function at all stages of endplate development by downregulating zDok-7 expression with antisense morpholinos. Our knockdown experiments demonstrate that zDok-7 is crucial for AChR prepatterning in zebrafish; these findings are similar to the findings in the Dok-7 knockout mouse, where no prepatterned AChR clusters were found in diaphragms of E14.5 mouse embryos (1). Prepatterned AChR clusters may be incorporated into forming neuromuscular synapses (15,17) but prepatterning is not a prerequisite for neuromuscular synapse formation (23).

Once the motor axon extends into the muscle, three types of innervation can be observed in zebrafish muscle: focal innervation in the centre of the myotome forming first, which is...
then replaced by distributed innervation via branched motor axons, and myoseptal innervation at the borders between individual somites (24). zDok-7 is not required for the formation of equatorial focal synaptic contacts. This differs from the unplugged zebrafish mutant, where no focal contacts form at all (24,27), implying that the signalling pathway leading to the initiation of synaptic contact in zebrafish is MuSK/unplugged—but not Dok-7 dependent.

Not all of the signalling pathways during different stages of NMJ formation require MuSK or Dok-7 to the same degree: Dok-7 seems less important for the formation of distributed and myoseptal AChR clusters—synaptic contacts do form but their appearance and number differ. The AChR clusters and synaptic contacts are smaller and less numerous at 48 hpf in Dok-7 morphants, indicating that Dok-7 determines the size, amount of branching and number of distributed synapses but not their formation. We observed a slight increase of myoseptal AChRs in Dok-7 morphants at 48 and 120 hpf compared with non-injected controls, further underlining differences in the signalling pathways controlling distributed and myoseptal innervation. However, the NMJs present in Dok-7 morphants are functionally impaired since the more severely affected morphants show motility defects.

The reduction in AChR cluster size and smaller size of neuromuscular synapses seen in zDok-7 morphants may be a primary consequence of zDok-7 deficiency or might be caused by aberrant AChR prepatternning. AChR prepatternning is not essential for synapse formation (23) but may be essential for synapses to reach appropriate size and shape by acting as scaffold for future sites of synaptic contact. UnpluggedFL MO-injected embryos have normal AChR prepatternning but later on axons fail to form contact with AChRs and all previously formed clusters disappear. At 48 hpf, almost no AChR clusters are detected in unpluggedFL morphants, but a phenotypic recovery is seen later in development. At 5 dpf an AChR staining pattern similar to zDok-7 MO-injected larvae is observed, with small punctate staining and increased myoseptal cluster localization (27). Similarly, ennui mutants have normal prepatternning, but smaller clusters at 48 and 120 hpf (26), indicating that AChR cluster size in focal and distributed synapses might not be determined by the presence of prepatternning.

Endplates of patients with DOK7 mutations showed a significant reduction in size of the postsynaptic area and simplified or degenerating synaptic folds (8,10). The Dok-7-deficient zebrafish model reiterates the finding of smaller endplates thereby confirming the significance of Dok-7 in defining endplate size. It would be interesting to examine endplates of Dok-7 mutant zebrafish beyond the age of 5 dpf up to adulthood in order to determine whether Dok-7 is involved in the maintenance or degeneration/regeneration processes at adult endplates.

Comparison with the mouse phenotypes of Dok-7 deficiency

The endplate phenotype caused by Dok-7 deficiency in zebrafish is less severe than the phenotype of the Dok-7 knockout mice. There could be several reasons for this: We cannot entirely exclude that a second Dok-7 orthologue is present in zebrafish, although BLAST database searches, of the previous zebrafish genome sequence assembly Zv7 and the most recent Zv8 assembly did not produce additional alignments. However, the zebrafish genome has not been completely sequenced. The puffer fish Takifugu rubripes, where the sequencing of the entire genome has already been completed,
has only one Dok-7 gene, reducing the likelihood that the Dok-7 gene has been duplicated in zebrafish. It might be possible that another protein is present at the zebrafish NMJ that can compensate for a lack of Dok-7; this protein either does not exist in mammals or has a different function. Equally, zebrafish Dok-7 might have a slightly different function in zebrafish as compared with mammals and may have different binding partners. Work by Hamuro and colleagues (12) identified two active SH2 target motifs in the COOH-terminal moiety of mammalian Dok-7. These SH2 target motifs are crucial for MuSK activation in myotubes (12). Although the PH-domain and PTB domain as well as first part of the C-terminal domain of Dok-7 are very well conserved among species, the rest of the C-terminal domain is more variable. The first of the two SH2 target motifs is missing in the zebrafish Dok-7 counterpart, thereby raising the possibility of different binding abilities of the C-terminal moiety of zebrafish Dok-7.

A last explanation for the mild endplate phenotype of Dok-7 deficiency in zebrafish could be an incomplete knockdown of Dok-7 expression by morpholino injection. This is difficult to investigate to date, as no antibody to control zDok-7 protein levels in Western blot or immunostainings is available. RT–PCR analysis of Dok-7 transcripts after knockdown with the splice morpholino showed exclusively mis-spliced transcripts but a low level of wild-type transcripts below the detection threshold might still be present and this could be sufficient to ameliorate the phenotype. A stable genetic Dok-7 null mutant would shed further light on this issue.

On the other hand, the zebrafish MuSK mutant unplugged also differs from the corresponding MuSK knockout mouse. The zebrafish mutant presents only a mild NMJ phenotype although no unplugged gene product is present. In contrast, the MuSK knockout mouse is not viable and forms no endplates at all (2). The zebrafish rapsyn mutant twitch once does not form distributed AChR clusters on the myotomal surface, only myoseptal AChRs are detected at 5 dpf; twitch once larvae are not able to perform a full touch escape response (28). No AChR clusters can be detected in the muscles of the rapsyn knockout mouse (3), as it is the case for the MuSK and the Dok-7 knockouts. A full comparison with the rapsyn mutant zebrafish is not appropriate in this case, as twitch once harbours a rapsyn missense mutation and not a null mutation. It may be that the differences seen between the mouse and the zebrafish models reflect the different innervation patterns of fish and mouse models and the difference in the requirement of the MuSK-Dok-7/rapsyn pathway for establishing the different types of neuro-muscular contacts. In mammals focal innervation by one motor axon to the central region of the muscle fibre is the most prevalent form of innervation. Non-focal innervation prevails in zebrafish tail muscles; focal innervation is only present at initial stages of endplate formation. MuSK and Dok-7 have not been investigated in mammalian muscles with non-focal innervation, e.g. extraocular muscles. Intriguingly, extraocular muscles are spared in the majority of DOK7 patients.

Dok-7 and muscle fibre morphology

In addition to changes at the NMJs we observed a disturbed muscle fibre structure from 48 hpf onwards, the time where touch escape response and free swimming should set in. The F59 labelled slow-muscle fibres are affected, instead of horizontal parallel alignment fibres have bowed appearance with a gap between neighbouring fibres. Slow muscle alignment looked normal at 24 hpf and lateral migration of slow muscle cells to the periphery was not affected by Dok-7 deficiency. We noticed a correlation of the appearance of slow fibres with the swimming abilities of morphants, as we saw the bowed fibre morphology almost exclusively in morphants with impaired swimming. In order to find out whether the changes in morphology are a primary consequence of the lack of Dok-7 or secondary effect caused by inactivity of the embryos we looked at slow muscle fibres of morphants with a similar NMJ phenotype. Downregulation of unplugged caused a more severe endplate phenotype with complete loss of distributed AChR clusters at 48 hpf and subsequently reduced motility. F59 slow muscle staining, however, was not altered in the unplugged morphants. This illustrates that slow fibre defects seen in Dok-7 morphants are not a cause of lack of distributed NMJs or of reduced amount of movement. Moreover, it implies that slow muscle mis-alignment in zDok-7 morphants is a direct consequence of lack of Dok-7 and this role of Dok-7 is independent of the MuSK/unplugged pathway.

Our results suggest that Dok-7 has an additional, previously unknown role in maintaining intact slow muscle fibre morphology. It is unclear, though, if this is a role unique to zebrafish muscle or if it is true also for mammals. Structure and arrangement of slow and fast muscle fibres are different in mammals and fish making it difficult to directly compare them; muscle fibre structure has not been investigated in the Dok-7 knockout mouse. In CMS patients with DOK7 mutations, muscle biopsies show unspecific changes consistent with a myopathy, e.g. type 2 fibre atrophy, type 1 fibre predominance and some regenerating fibres (5,7,8). More detailed fibre-type staining on longitudinal muscle sections or ultrastructural muscle analysis might provide more information on muscle fibre integrity in patients.

Our work in zebrafish confirms the findings from the studies of patient endplates that Dok-7 influences NMJ structure and size. Thus zebrafish represents a good model for DOK7 CMS regarding disease features and severity. In general, zebrafish may be a good alternative to mice as animal model for NMJ disorders: early embryonic stages of NMJ development are well established and are all readily accessible for imaging. The protein composition of NMJ in zebrafish is quite similar to the human. Further advantages of the zebrafish model lie in the possibility to easily manipulate protein expression by RNA or oligonucleotide injection, and to obtain a large number of embryos and adult animals in a short period of time. Zebrafish NMJ mutants could be used for screens to identify compounds with a beneficial effect for CMS patients. In contrast, early lethality of NMJ protein knockout in mice decreases the utility of this animal model for drug screening.
Our results in zebrafish point towards an additional function of Dok-7 in maintaining myofibre structure. A complex Dok-7 function that extends beyond the NMJ and its possible involvement in multiple signalling pathways in muscle might explain why findings at patients’ endplates are not reiterated in tissue culture models looking only at the AChR clustering abilities of Dok-7 mutants. If this additional function is also established in humans it might help to understand the clinical findings in patients. Clinical features of DOK7 patients are more reminiscent of a myopathy or limb-girdle muscular dystrophy than of CMS and are very different from clinical features of RAPSN or MUSK patients (5–7). A reduced AChR number at endplates or small endplates does not explain the non-responsiveness to esterase inhibitor therapy observed in DOK7 patients either; this would be more in accordance with an additional myopathic component of DOK7 CMS and our results offer a potential route to explore this.

MATERIALS AND METHODS

Fish strains and maintenance

The wild-type strain *AB was used in this study (Zebrafish International Resource Centre (ZIRC), Oregon). Zebrafish embryos were collected and raised at 28.5°C according to standard procedures (29) and staged in hours or days post-fertilization (hpf or dpf) according to standard criteria (19). Touch-evoked swimming response in 48 hpf embryos was elicited by touching the head or the tail of the embryos with a fine pipette tip. Movements of embryos and larvae were recorded using a Moticam 1000 camera mounted on a dissection stereomicroscope.

Antisense morpholino oligonucleotide injection

Antisense morpholino oligonucleotides (MO, Gene Tools LLC) targeted to interfere with zDok-7 translation or splicing were designed using the zebrafish Dok-7 mRNA sequence (LOC557881, mRNA accession number XM_681035) and the corresponding genomic DNA sequence obtained from zebrafish chromosome 1 assembly Zv7_scaffold49, accession number NC_007112).

The following two morpholinos targeting zDok-7 transcripts were used: zDok-7 translation-blocking MO1: 5′-CTCTACGA CAACCGTATCCGTCATC and zDok-7 exon 2 splice MO2: 5′-ATTTATAGGATTTACCTGCTACCGG. The splice morpholino is directed against the splice donor site of exon 2, leading to skipping of the exon and consequently to a frameshift and premature translation termination after 25 missense amino acids.

As control MO we used the Gene Tools standard control MO: 5′-CCTCTTACCTCACGTAAAAATATA-3′.

Morpholinos were resuspended in 1× Danieau solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO4, 0.6 mM Ca(NO3)2, 5 mM HEPES; pH 7.6) with phenol red as an injection indicator. Morpholinos were injected into the yolk of one-to four-cell stage embryos. Embryos were injected with between 15 ng and 20 ng of zDok-7 and control morpholino and 2–6 ng of unpluggedFL morpholino as specified by Zhang et al. (27). Light microscope images of injected and non-injected embryos were taken with a digital camera (model LEICA DFC 420C) mounted on a Leica dissection stereomicroscope.

RNA isolation, RT–PCR

RNA from zebrafish embryos, larvae and adult tail tissue was isolated with Trizol reagent (Invitrogen) following the manufacturer’s instructions. For RT–PCR analysis following morpholino injection, RNA from approximately 30 embryos was extracted and after cDNA synthesis, PCR using gene-specific primer pairs was performed: zDok-7: 5′-UTR forward: 5′-AGGCCGGACCTCAGTAATTTC and exon3 reverse: 5′-TTTACTCCATCAAGCCCTGAAGA; for unpluggedFL: 5′-AAACGAGTCTGCCATGT-3′ and 5′-ATGAACTGACCCGTC-3′ (27).

The same primer pair as above was used for semi-quantitative analysis of zDok-7 expression at different developmental stages. Reverse transcription was performed using the Superscript III First-Strand Synthesis System (Invitrogen); MolTaq DNA polymerase (Molzym) was used for the PCR reactions.

Elongation factor 1α was used as control transcript as it is known to be expressed at similar levels throughout all developmental stages (30), whereas AChR α1 served as control for an NMJ gene. Primer pairs:

EF1α: forward: 5′-CTGGAAGGACGGCTCAAACATGG and reverse: 5′-CTTCTTGCTCTCCAGACCCACATTAC AChR α1: forward: 5′-AGGCCGGACCTCAATATC and reverse: 5′-ATTATGAGGAACGGACTGAGAA.

Whole-mount antibody immunofluorescence stainings

For whole-mount immunofluorescence staining, embryos were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature for 2 h and then permeabilized in cold acetone at −20°C. Depending on their age, 3–5 dpf old larvae were permeabilized with collagenase A (Roche Diagnostics, 1 mg/ml) for 30–90 min. Embryos/larvae were blocked in 5% horse serum in PBS containing 0.1% Tween-20 (PBT). Embryos/larvae were incubated in blocking solution containing primary antibody overnight at 4°C followed by washing several times with PBT and incubation with secondary antibody (goat anti-mouse Alexa Fluor® 488, or donkey anti-mouse Alexa Fluor 594, Invitrogen). The following primary antibodies were used in our study: SV2 (1:200), F59 (slow muscle myosin light chain, 1:50) and F310 (fast muscle myosin heavy chain, 1:200) (all from Developmental Studies Hybridoma Bank, Iowa). AChRs
were visualized by using Alexa Fluor 594 conjugated α-bungarotoxin (1 μg/ml, Invitrogen). Fluorescein isothiocyanate (FITC) conjugated phalloidin (Sigma) was used at a concentration of 5 μg/ml to label actin.

Embryos were imaged using a Zeiss LSM510 laser scanning confocal microscope. Z-stack images were generated by scanning through one half of the myotome of the trunk using a 20× or a 40× oil objective.

For the quantification of AChR clusters, we scanned somites of 10 48 hpf non-injected embryos and of 10 Dok-7 MO-injected embryos that have been stained with Alexa Fluor 594 conjugated α-bungarotoxin (20× objective, one image per embryo, around somites 9–16). We used Image J software to measure the total α-bungarotoxin positive area as fraction of the total tail area.

**In situ hybridization**

Whole-mount in situ hybridization was performed according to the standard procedures described in (31,32). PCR primers used for generating zDok-7 in situ hybridization probes were: exon 6 forward: 5′-ggataacagagagctgtcagtg and exon 7 reverse: 5′-acacataaaaaacctttggaatgac (yielding a 926 bp PCR fragment) or exon 10 reverse: 5′-gaatcaccttgagc (yielding a 1063 bp PCR fragment). PCR fragments were cloned into pGEM-T Easy vector (Promega); antisense and sense control probes were transcribed with T7 RNA polymerase; fragments were cloned into pGEM-T Easy vector (Promega); sense and antisense probes were transcribed with T7 RNA polymerase, respectively, and labelled using the DIG RNA labelling kit (Roche Diagnostics). The hybridization temperature was 65°C for both probes.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**ACKNOWLEDGEMENTS**

We would like to thank the staff of the Institute of Human Genetics Zebrafish Facility for expert fish care, Drs Bill Chaudhry and Nicholas Child for experiment advice and help with the in situ hybridization, and Lisa Hodgson for help with microscope imaging.

**Conflict of Interest statement:** None declared.

**FUNDING**

This work was supported by a grant from the Association Française contre les Myopathies (AFM) to H.L., C.J. and J.S.M., by Science City Newcastle, and by the MRC (UK) as part of the MRC Centre for Neuromuscular Diseases. J.S.M. received a fellowship from the Deutsche Forschungsgemeinschaft (MU2840/1-1). K.B., V.S. and H.L. are members of TREAT-NMD; Newcastle University is the coordinating partner of the TREAT-NMD network of excellence (EC, 6th FP, proposal #036825; www.treat-nmd.eu).

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