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Eif4a3 is required for accurate splicing of the *Xenopus laevis ryanodine receptor* pre-mRNA

Tomomi Haremaki, Daniel C. Weinstein *

Queens College of the City University of New York, Biology Department, 65–30 Kissena Boulevard, Flushing, NY 11367, USA

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ABSTRACT

The Exon Junction Complex (EJC) plays a critical role in multiple posttranscriptional events, including RNA subcellular localization, nonsense-mediated decay (NMD), and translation. We previously reported that knockdown of the EJC core component Eukaryotic initiation factor 4a3 (Eif4a3) results in full-body paralysis of embryos of the frog, *Xenopus laevis*. Here, we explore the cellular and molecular mechanisms underlying this phenotype. We find that cultured muscle cells derived from Eif4a3 morphants do not contract, and fail to undergo calcium-dependent calcium release in response to electrical stimulation or treatment with caffeine. We show that *ryr* (*ryanodine receptor*) transcripts are incorrectly spliced in Eif4a3 morphants, and demonstrate that inhibition of *Xenopus* Ryr function similarly results in embryonic paralysis. These results suggest that the EJC mediates muscle cell function via regulation of pre-mRNA splicing during early vertebrate embryogenesis.

Introduction

RNA processing plays a fundamental role in the regulation of gene expression (Moore and Proudfoot, 2009). Recent studies have implicated the multi-protein Exon Junction Complex (EJC), which binds upstream of exon splice junctions in a sequence-independent fashion, in a number of RNA processing events, including localization, splicing, translation, and degradation (Le Hir et al., 2001; Le Hir et al., 2000; Tange et al., 2004). The EJC consists of several core proteins and numerous accessory factors (Bono and Gehring, 2011; Moore and Proudfoot, 2009); Eif4a3 is the EJC core component thought to directly contact RNA (Ballut et al., 2005; Shibuya et al., 2004). Eif4a3 was first identified in animal cells in a screen for transcripts upregulated in the ventral ectoderm of gastrula stage *Xenopus* embryos (Weinstein et al., 1997). Eif4a3 expression is quite dynamic during subsequent stages of development, suggesting diverse, tissue-restricted activities for this factor (Weinstein et al., 1997). Eif4a3 misexpression is sufficient to drive epidermal induction in cells otherwise destined to adopt a neural fate (Weinstein et al., 1997). Loss-of-function studies suggest that the requirement for Eif4a3 during development is more nuanced: morpholino-mediated knockdown of Eif4a3 leads to defects in heart looping and melanophore development and, strikingly, complete embryonic paralysis (Haremaki et al., 2010).

Although it is clear that Eif4a3 is essential for normal embryogenesis, the mechanisms underlying this requirement are not well understood. Knockdown of the EJC core components Y14 and Magoh give rise to defects similar to those seen in Eif4a3 morphants, suggesting that the embryonic requirement for Eif4a3 is mediated through its role in the EJC (Haremaki et al., 2010; Kenwrick et al., 2004); however, the pleiotropic effects seen following Eif4a3 loss-of-function may reflect the disruption of one or several distinct RNA processing functions for this protein, acting on one or more target transcripts.

In order to address the specific requirements for Eif4a3 during development, we have focused on one aspect of the Eif4a3 morphant phenotype: we describe here the cellular and molecular mechanisms underlying the paralysis that results from Eif4a3 knockdown. We find that muscle cell cultures derived from Eif4a3 morphant embryos fail to contract in response to electrical or chemical stimulation; this appears to result from a defect in calcium-dependent calcium release. We demonstrate that the Ryanodine receptor 1 (Ryr1), a key mediator of intracellular calcium release, is required for embryonic movement in *Xenopus*; furthermore, we show both that Ryr1 is dramatically downregulated following Eif4a3 knockdown, and that *ryr1* transcripts are improperly spliced in Eif4a3 morphants. Our results thus implicate Eif4a3 in muscle cell function, via regulation of *ryr1* pre-mRNA splicing.

Materials and methods

Eif4a3 constructs and morpholinos

For Myc–Eif4a3, the coding sequence of *X. laevis eif4a3* was amplified by PCR using the following primers: XEif4a3–5mycU: AGGCCTGCGGCCGCAGCTGTTGCAG; XEif4a3–5mycD: AGGCCTCA

* Corresponding author. Fax: +1 718 559 6745.
E-mail address: daniel.weinstein@qc.cuny.edu (D.C. Weinstein).

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AATAAGATCAGCAACGTTC and subcloned downstream of six Myc (EQKLISEEDLNEM) epitopes in the StuI site of CS2+MT. *X. laevis* Eif4a3 morpholino (Eif4a3MO) and the five-mismatch control morpholino (MM) were described previously (Haremaki et al., 2010). A construct containing only the coding sequence of *X. laevis* Eif4a3 was described previously (Weinstein et al., 1997); both this and the Myc–Eif4a3 construct lack the Eif4a3MO-binding site.

Muscle cell culture

X. laevis muscle cell cultures were prepared as described (Nacira Tabti, 1998). For electrical charge assays, cells were stimulated directly by silver electrodes at 10 V, 20 V, or 200 V 6 Hz using an Isolated Pulse Stimulator (model# 2100, A-M Systems). Immunocytochemistry for *Xenopus* muscle cell culture was performed as described (Campbell et al., 2006). Anti-myosin antibody (MF20, Developmental Studies Hybridoma Bank) was used at 1:60 dilution; FITC-conjugated secondary antibody (Jackson ImmunoResearch) was used at 1:50 dilution.

Calcium imaging

Stock solution (1 mM) of the membrane-permeant form of fluo-4 (Invitrogen) was prepared in DMSO, then added to culture media to give a final concentration of 3 μM. The nonionic detergent Pluronic F-127 (0.03% final) was added to increase solubility (Prada et al., 2005). Two days after culture preparation, cells were incubated with fluo-4 for 30 min at room temperature, then washed 3 times in culture media. 10 V 6 Hz 2 s electrical stimulation was applied to the cells; simultaneously, images of fluo-4 treated cells were taken by 2 s exposure using a Leica DMIL inverted microscope with filter module I3 and a DFC420c camera, and analyzed by ImageJ.

Caffeine and ionomycin treatment

For caffeine treatment, stock solutions (300 mM) were diluted into 62.5 mM in 1.2 mL of culture medium removed from the cultured cells; this was then added back to the remaining 1.8 mL medium. For ionomycin treatment, stock solutions (2 mM) were diluted into 200 μM with PBS then added to culture medium at a concentration of 1:100.

Ryanodine receptor genomic sequence and splice-blocking morpholinos

X. laevis partial *ryr1* cDNA sequence (Contig043167) was obtained from XDB3.2 (http://xenopus.nibb.ac.jp/). To acquire *ryr1* genomic sequence, *X. laevis* genomic DNA was amplified by PCR using the following primers: XlRYRprobeU: TCTGTCCATTCTGGGACACT,

XlRYRprobeD: GACCAGTGTGTTCCGTTTCA. The amplified 3.2 kb fragment was cloned into the T-vector (Promega) and fully sequenced. Exon-intron structures were predicted by comparison with *Xenopus tropicalis ryr1* (JGI v4.1, ID: 469327). A splice-blocking morpholino (RyrMO)(AGATAATGTTCTCTGACCTGTTTGC) was designed at the exon 99-intron 99 boundary. The effects of this morpholino on *ryr1* transcripts were confirmed by RT-PCR using the following primers: XlRYRprobeU: TCTGTCCATTCTGG-GACACT, RYRnewD: CAGCTCTCCAAATGCATCAA. The Ryr5MM morpholino (AGAaAATcTTgTCTcACCTcTTTGC) introduced 5 base pair mismatches to the RyrMO sequence.

RNA co-immunoprecipitation (RIP) and RT-PCR

1 ng *Myc–eif4a3* RNA was injected with and without 21 ng Eif4a3MO into 2-cell stage embryos, which were subsequently harvested at stage 27. RNA co-immunoprecipitation assays were performed as described (Vishnu et al., 2011). Precipitated RNA was purified by RNA-Bee (Tel-Test, Inc.). After DNase treatment, cDNA was synthesized with random hexamers by MMLV Reverse Transcriptase (Promega). RT-PCR was performed as described (Wilson and Hemmati-Brivanlou, 1995). Primers used in this study are as follows:

Xlryr5′F1: GAGGAGATCCAGTTCCTCAG,
Xlryr5′R4: GAATAGCATGGCCATAGAGC,
RYRnewU: CTGGCCGTGGTTGTTTATCT,
RYRnewD: CAGCTCTCCAAATGCATCAA,
ProbeD: GACCAGTGTGTTCCGTTTCA,
RYRE101F: TGCTATCTCTTCCACATGTA (exon 101 primer),
RYRI101F: GAACCCGAAAATACCCCATC (intron 101 primer),
RYRE102R: CTCCATGTCCTCCTTTACTTG (exon 102 primer),
FoxD3U: TGTGGAGCGTAACTGGAATG,
FoxD3D: GTTCTTGGGCTTGTTCTGGA,
GSK3BP_U: TTCTTGCGTGAGGGGTAGAA,
GSK3BP_D: CATTGCACGGTTGTCTCAGT,
CYP1C_U: GCCCCATCTCACCTTTTGTA,
CYP1C_D: GAAGTCAAGCGCAGGAAAAC,
ODC-F: AATGGATTTCAGAGACCA,
ODC-R: CCAAGGCTAAAGTTGCAG.

Western blot analysis

Western blot analysis was performed as described (Hama et al., 2002). Antibodies that detect both *ryanodine receptor-1* and *-2* isoforms (34C, Developmental Studies Hybridoma Bank) were used at 1:50 dilution. Antibodies against β-Tubulin (T8660, Sigma) were used at 1:200 dilution. Secondary antibodies (donkey anti-mouse IgG coupled to horseradish peroxidase) (Jackson Immuno Research) were used at 1:1,000 dilution.

Results

Eif4a3 is required for muscle cell contraction

We previously reported that knockdown of the EJC component Eukaryotic initiation factor 4a3 (Eif4a3) results in full-body paralysis in embryos of the frog *Xenopus laevis* (Haremaki et al., 2010). Paralysis can arise from a variety of causes, including defects in skeletal muscle and/or neuronal development. Eif4a3 morphants have sensory neuron defects that may underlie the lack of touch response in these embryos; however, this is not a likely cause of paralysis (Haremaki et al., 2010). While we observed no marked differences in somite structure between Eif4a3 morphants and control embryos, we have not yet addressed directly the effects of Eif4a3 knockdown on muscle cell activity.

To investigate the potential requirement for Eif4a3 in muscle development and function, muscle cell cultures were prepared from stage 23 dorsal explants of uninjected embryos, Eif4a3 morpholino (Eif4a3MO)-injected embryos, or embryos injected with a control morpholino that differs at 5 base pairs and does not affect Eif4a3 translation (Eif4a3MM) (Haremaki et al., 2010; Nacira Tabti, 1998). Spindle-shaped cells obtained from control and morphant embryos were morphologically indistinguishable; staining with the anti-myosin specific antibody MF20 confirmed that these were indeed muscle cells (Fig. 1A)(Bader et al., 1982). To assess muscle cell contractility, we subjected cultures to electrical stimulation (Xie et al., 1997). All Eif4a3MM-injected spindle-shaped muscle cultures tested contract in response to electrical stimulation of 20V (100%; $n=34$); muscle cultures derived from Eif4a3MO-injected