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Smooth muscle caldesmon modulates peristalsis in the wild type and non-innervated zebrafish intestine

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Abstract

Background—The high molecular weight isoform of the actin-binding protein Caldesmon (h-CaD) regulates smooth muscle contractile function by modulating cross-bridge cycling of myosin heads. The normal inhibitory activity of h-CaD is regulated by the enteric nervous system; however, the role of h-CaD during intestinal peristalsis has never been studied.

Methods—We identified a zebrafish paralog of the human CALD1 gene that encodes an h-CaD isoform expressed in intestinal smooth muscle. We examined the role of h-CaD during intestinal peristalsis in zebrafish larvae by knocking down the h-CaD protein using an antisense morpholino oligonucleotide. We also developed transgenic zebrafish that express inhibitory peptides derived from the h-CaD myosin and actin-binding domains, and examined their effect on peristalsis in wild-type zebrafish larvae and *sox10^{colourless}* mutant larvae that lack enteric nerves.

Key Results—Genomic analyses identified two zebrafish Caldesmon paralogs. The *cald1a* ortholog encoded a high molecular weight isoform generated by alternative splicing whose intestinal expression was restricted to smooth muscle. Propulsive intestinal peristalsis was increased in wild-type zebrafish larvae by h-CaD knockdown and by expression of transgenes encoding inhibitory myosin and actin-binding domain peptides. Peristalsis in the non-innervated intestine of *sox10^{colourless}* larvae was partially restored by h-CaD knockdown and expression of the myosin-binding peptide.

Conclusions & Inferences—Disruption of the normal inhibitory function of h-CaD enhances intestinal peristalsis in both wild-type zebrafish larvae and mutant larvae that lack enteric nerves, thus confirming a physiologic role for regulation of smooth muscle contraction at the actin filament.

Keywords

enteric nervous system; gastrointestinal motility; hypomotility; myosin light chain; zebrafish

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AUTHOR CONTRIBUTIONS

JA, GD, and CS performed the experiments, and all authors contributed to analysis and interpretation of the data; JA and MP conceived the study and designed the experiments; JA and MP drafted the manuscript.

DISCLOSURES

No competing interests declared.

INTRODUCTION

Peristalsis in the mammalian intestine is regulated by the enteric nervous system (ENS). ENS stimulation initiates smooth muscle contraction through a calcium-mediated signaling cascade that culminates in phosphorylation of the regulatory Myosin light chain (Mlc) which is physically associated with the myosin filament.^{1,2} Phosphorylation of Mlc by Myosin light chain kinase (Mlck) induces a conformational change in myosin that activates its ATPase. This induces cross-bridge cycling within the actomyosin complex, and ultimately, the generation of contractile force. Biochemical analyses have shown that endogenous actomyosin interactions can generate and sustain smooth muscle contractile force when levels of phospho-Mlc are greatly reduced.³⁻⁶ To account for this, it has been proposed that contraction is also regulated by actin-binding proteins that alter actomyosin interactions independent of phospho-Mlc.⁷

One smooth muscle actin-binding protein that has been extensively studied is Caldesmon (CaD).⁸ CaD exists as two predominant isoforms that are generated by alternative splicing of a single mRNA transcript.⁹ The low molecular weight isoform (l-CaD) is expressed in most cell types, including at low levels in smooth muscle, where it mediates actin and non-muscle myosin interaction in the cortical cytoskeleton.¹⁰ The high molecular weight isoform (h-CaD) is expressed specifically in smooth muscle. It is distinguished from l-CaD by a peptide spacer domain in the middle of the protein that is thought to enable h-CaD's simultaneous binding to smooth muscle actin and myosin filaments.¹¹ Binding of h-CaD to the phosphorylated actomyosin complex reduces contractile force by inhibiting myosin ATPase activity,¹²⁻¹⁴ possibly by restricting myosin binding to actin, or by stabilizing a less active configuration of the actin filament¹⁵ (Fig. 1A based on prior model¹⁶). h-CaD's inhibition of the smooth muscle actomyosin complex can be reversed by phosphorylation or through its interaction with Calmodulin in the presence of calcium.¹⁷⁻¹⁹ This results in increased contractile force by enhancing myosin binding to actin²⁰ (Fig. 1B), which in turn may stabilize an active configuration of the actin filament.¹⁵ In smooth muscle with low levels of phospho-Mlc, phospho-h-CaD is thought to enhance contraction by promoting interaction of non-phosphorylated myosin heads with actin²¹ (Fig. 1C,D).

Both the inhibitory role of h-CaD and the molecular signaling pathways that regulate its activity have been characterized in smooth muscle cells from the digestive tract and other mammalian tissues.^{13,22-26} Despite these advances in understanding h-CaD function and regulation, its role in modulating complex physiologic responses, such as intestinal peristalsis, has not been directly examined *in vivo*. Isoform specific knockout of h-CaD in mice has been reported; however, its effect on smooth muscle function was not examined.²⁷ Here, we address the role of h-CaD in intestinal smooth muscle *in vivo* using the zebrafish system. We show that the zebrafish genome contains two Caldesmon gene paralogs, *cald1a* and *cald1b*, that are orthologs of the human *CALD1* gene. The *cald1a* paralog encodes an h-CaD isoform expressed in intestinal smooth muscle. Disruption of h-CaD function enhances propulsive peristalsis in wild-type zebrafish larvae and in *sox10^{colourless}* mutants that lack enteric nerves thus confirming an *in vivo* regulatory role for h-CaD in smooth muscle with normal and low levels of phospho-Mlc.

MATERIALS AND METHODS

Fish stocks

Maintenance and breeding were performed as previously described.²⁸ Colourless mutants were purchased from the Zebra-fish International Resource Center (Eugene, OR, USA).

Morpholino knockdown

Fertilized embryos were injected at one-cell stage with 20 pg of the *Cald1a-i4e5* morpholino. (Data S1).

RT-PCR

RNA was isolated from dissected 5 days post fertilization (dpf) larval intestines, and RT-PCR was carried out using standard procedures (Data S1). Primer sequences are listed in Table S1.

Generation of CALD1 myosin- and actin-binding peptide transgenic fish

Primers corresponding to the human *CALD1* myosin-binding peptide²⁹ were identified in the zebrafish *cald1c*DNA. Transgenic constructs were generated with the peptide fragment by Tol2kit site-specific recombination-based cloning (Data S1).

Intestinal microsphere assay

Larvae were fed paramecia in media containing fluorescent latex beads, and bead motility was monitored (Data S1).

Cell dissociation and FACS

Dissociation and cell sorting were performed using standard procedures (Data S1).

Western blot/ immunoprecipitation

Dissected intestines from 30 larvae at 4 dpf and 6 dpf were homogenized in 1× sample buffer, and protein was recovered and used for Western analysis using standard methods³⁰ (Data S1).

RESULTS

The zebrafish genome contains two gene paralogs with high sequence homology to human CALD1

To identify a zebrafish h-CaD ortholog, we first performed BLAST analyses of the most recent zebrafish genome assembly using human *CALD1* coding sequence. Two loci encoding genes with relatively high sequence homology to *CALD1* were identified on chromosome 4 and chromosome 25 (hereafter *cald1a* and *cald1b*, respectively). The predicted protein encoded by the *cald1a* locus had the greatest degree of homology to human CALD1, particularly in the actin and myosin-binding domains (Fig. 2A, and Figure S1).

We also compared syntenic relationships surrounding the *cald1a* and *cald1b* loci with human *CALD1*. We first identified the map positions of the zebrafish orthologs of 65 genes immediately surrounding human *CALD1* (Fig. 2B, and Figure S2). Of 34 genes located 5' of *CALD1*, 26 were located in a comparable position with respect to the *cald1a* locus, whereas only six mapped to a comparable position with respect to *cald1b*. For 31 genes 3' of *CALD1*, 14 were located surrounding *cald1a*, whereas none were located adjacent to *cald1b*. Next, we performed the reciprocal experiment and mapped the position of the human orthologs of genes surrounding the two zebrafish *cald1* loci. Twelve of 40 genes 5' of *cald1a*, but only 7 of 38 genes 5' of *cald1b* mapped to comparable regions of *CALD1*. Similarly, nine of 43 genes 3' of *cald1a*, but only 3 of 38 genes 3' of *cald1b* mapped nearby *CALD1*. Paralogs for 17 zebrafish genes were identified in the region surrounding the two *cald1* loci (Figure S2).

All together, these findings argue that *cald1a* and *cald1b* are gene paralogs that arose from a whole genome duplication which occurred in an ancestral species of ray-finned fish.³¹ We predicted that the Cald1a protein was likely to be a functional ortholog of human CALD1 protein based on amino acid sequence homology with CALD1, and because of more highly conserved gene synteny surrounding the *cald1a* locus.

cald1a encodes high and low molecular weight isoforms generated by alternative splicing

The *cald1b* locus encodes an l-CaD-like ortholog that was recently reported to play a role in cardiovascular development.³² Genomic sequence analysis of this locus did not identify a potential h-CaD transcript nor did BLAST searches of the zebrafish EST database. To determine whether an alternatively spliced h-CaD mRNA transcript was encoded by *cald1a*, we amplified its full length cDNA from whole 5 dpf larvae that have functional intestinal smooth muscle. These primers amplified only a fragment corresponding to the predicted low molecular weight *cald1a* isoform (data not shown). When the same primers were used to amplify RNA recovered from dissected intestines (that were enriched for smooth muscle) the low molecular weight band and a higher molecular weight band were recovered (Fig. 2C). In contrast, amplification of intestinal cDNA using comparable primers from the *cald1b* ortholog only amplified low levels of a corresponding low molecular weight band (Fig. 2C). DNA sequence analysis showed that the larger band amplified from *cald1a* encoded a predicted h-CaD ortholog that included an 86 amino acid spacer domain. Genomic DNA analyses indicated that the spacer was encoded by two exons, 36 basepairs (bp) and 225 bp respectively. We observed only limited amino acid homology with spacer domains from other vertebrate h-CaD proteins (Fig. 2A, and Figure S1). This was expected, as the spacer sequence is not highly conserved in human, mouse or chicken h-CaD. Outside of the spacer domain, the h-CaD cDNA sequence was identical to the full length *cald1a* cDNA amplified from RNA derived from whole larvae, although a splice variant involving the terminal exon was infrequently detected (Figure S3). These findings show that the *cald1a* locus encodes two alternatively spliced transcripts that are orthologs of the mammalian l-CaD and h-CaD isoforms.

To determine whether the h-CaD transcript encoded a functional protein, we performed Western blot analysis of 5 dpf intestinal extracts using an antibody directed against mouse CaD protein. This antibody detected both high and low molecular weight bands (Fig. 2D). The high molecular weight band was first detected around the onset of peristaltic contractions (~78 h post fertilization; data not shown) when only circular smooth muscle is present,²⁸ and protein levels increased between 4 dpf and 6 dpf. At these later stages, longitudinal smooth muscle develops, and peristaltic contractions are more pronounced.²⁸ The estimated molecular mass of the high molecular weight band (125 kD) was significantly greater than the predicted h-CaD molecular mass (70 kD). A comparable discrepancy between predicted and observed molecular mass of mammalian h-CaD proteins has been reported and attributed to its relatively large number of acidic amino acids.³³ The molecular weight of the presumptive l-CaD protein was similarly affected.

h-CaD expression in the zebrafish intestine is restricted to smooth muscle

High molecular weight Caldesmon isoform expression in mammals and chicken is restricted to smooth muscle, whereas l-CaD is ubiquitously expressed. RNA *in situ* hybridization using an isoform specific zebrafish h-CaD probe showed only low level background staining in 5 dpf larvae (data not shown). To assay h-CaD expression in intestinal smooth muscle, we recovered epithelial cell and smooth muscle cell RNAs from bigenic larvae that express cell-type specific fluorescent reporters (Fig. 3A). The bigenic larvae express GFP from the smooth muscle *sm22a* promoter beginning around 72 h post fertilization³⁴ and mCherry from a *miR194* promoter fragment that is activated in the epithelium beginning around the

same time. RT-PCR amplification of established epithelial (*fabp2*, *vill1*, and *fabp6*) and smooth muscle (*acta2*, *myh11*) markers confirmed purity of the sorted cell populations (Fig. 3B). The RT-PCR experiments also confirmed that zebrafish h-CaD expression was restricted to smooth muscle, whereas l-CaD was expressed in both smooth muscle and epithelial cells (Fig. 3C).

Knockdown of zebrafish h-CaD enhances propulsive intestinal peristalsis

To investigate zebrafish h-CaD function *in vivo*, we generated a *cald1a* splice blocking morpholino (Cald1a-i4e5 MO) that enabled us to specifically disrupt h-CaD translation without affecting translation of the l-CaD isoform. The specificity of Cald1a-i4e5 MO also prevented any disruption of the normal function of *cald1b*. It was crucial not to disrupt l-CaD function as this isoform is predicted to be essential for a wide range of non-muscle phenotypes. For this study, we designed a morpholino complementary to the exon 5 splice acceptor of the h-CaD pre-mRNA (Fig. 4A). This was predicted to generate a novel Caldesmon transcript that encoded only 12 of 87 h-CaD spacer region amino acids.

Zebrafish embryos and larvae injected with Cald1a-i4e5 MO had normal morphology other than mild developmental delay that was also seen in control larvae (Fig. 4B). In RT-PCR experiments using intestinal RNA and primers surrounding exons 4 and 5, a fragment of the truncated h-CaD transcript that lacked exon 5, but retained exon 4, and the fragment corresponding to l-CaD were both amplified (Fig. 4C). The truncated h-CaD transcript, which was 36 bp larger than endogenous l-CaD transcript, encoded 12 amino acids in-frame with exon 6. Successful inhibition of translation of the h-CaD transcript was confirmed using Western blot (Fig. 4D). The apparent increase in l-CaD protein detected by this blot is likely due to the presence of the truncated h-CaD protein generated by the Cald1a-i5e5 MO (as it is only 12 amino acids longer than endogenous l-CaD).

To determine whether h-CaD knockdown affected smooth muscle contraction, we assayed propulsive peristalsis in the intestine of control and Cald1a-i4e5 MO injected larvae that were fed paramecia combined with fluorescent microspheres (Fig. 4E). Following their ingestion, the microspheres form an aggregate in the intestinal bulb as a result of mixing peristalsis. Thereafter, the aggregate is transported into the mid and posterior intestine via propulsive peristalsis, and eventually expelled. We measured peristalsis in 4 dpf and 5 dpf control and Cald1a-i4e5 MO injected larvae 2 h after bead ingestion. As shown in Fig. 4E, a higher percentage of h-CaD deficient larvae had transported beads to the mid-posterior intestine and/or expelled the beads. Video recordings of live wild type and Cald1a-i4e5 MO injected larvae showed that the rate of peristaltic contractions was unaffected by h-CaD knockdown (Figure S4). The effect of Cald1a-i4e5 MO on peristalsis was less pronounced at 5 dpf than at 4 dpf, most likely because of the transient nature of the morpholino knockdown. In addition, because levels of phospho-h-CaD have increased at this stage,³⁰ the overall level of inhibition imparted by h-CaD is likely to be reduced. Together these data indicate that the normal function of h-CaD at this developmental stage is to negatively regulate the force of phasic intestinal smooth muscle contraction, as intestinal transit is accelerated in the larvae injected with Cald1a-i4e5 MO without a concomitant effect on contractile rate.

Interference with h-CaD binding to smooth muscle myosin and actin enhances intestinal propulsive peristalsis

h-CaD tethers myosin and actin filaments through interactions with its N- and C-terminal domains, respectively.^{35,36} The h-CaD – smooth muscle myosin interaction can be disrupted *in vitro* by expression of cDNA encoding a peptide fragment from the myosin-binding domain common to l-CaD and h-CaD isoforms.²⁹ By disrupting endogenous h-CaD binding

to myosin, actin–myosin interactions are enhanced, thereby increasing basal contractile tone. A comparable effect has been reported with a peptide derived from the CaD actin-binding domain.^{20,37}

We generated a transgenic line, *Tg(sm22a:CADDK51-GFP)*, with stable smooth muscle expression of a zebrafish ortholog of mammalian myosin-binding peptide (CADDK51) to assay h-CaD function in older zebrafish larvae that have a well-developed enteric neuromuscular system. This was not possible in larvae injected with the *Cald1a-i4e5* MO, because its inhibitory effect on translation is transient. In addition to the cDNA encoding the peptide, the expression vector used to generate the transgenic line encoded GFP downstream of a viral 2A recognition motif.³⁸ This allowed us to identify fluorescent smooth muscle cells expressing the CADDK51 peptide following its cleavage from GFP. F₀ larvae that had mosaic expression of CADDK51 in smooth muscle under control of the zebrafish *sm22a* promoter and F₁ germline transgenic progeny with ubiquitous intestinal smooth muscle GFP expression were both viable (Fig. 5A). Variable transgene expression was detected in F₁ larvae, most likely resulting from position effects arising from independent insertion sites of the trans-gene into the F₀ germline. Collectively, F₁ transgenic larvae were used to further assay the *in vivo* function of h-CaD in intestinal smooth muscle.

The effect of CADDK51 on h-CaD–myosin filament interaction was examined by measuring levels of h-CaD bound to smooth muscle myosin protein (Myh11) in the transgenic larvae (Fig. 5B). As h-CaD remains bound to Acta2 in the actomyosin complex, transgenic larvae injected with a morpholino targeting smooth muscle actin protein (Acta2)³⁰ were used for this immunoprecipitation experiment. This allowed us to determine the effect of CADDK51 on h-CaD–myosin interaction independent of h-CaD–Acta2 interaction. Indeed, Western blot analysis showed that there was significantly less h-CaD bound to Myh11 in Acta2 deficient transgenic larvae compared with control transgenic larvae. These findings support binding of the peptide fragment to Myh11 with subsequent displacement of endogenous h-CaD.

To determine the effect of CADDK51 peptide on smooth muscle contraction *in vivo*, we assayed intestinal peristalsis in the transgenic larvae. Using the intestinal microsphere assay, we found a significant increase in the propulsive peristalsis of 6 dpf F₁ transgenic larvae compared with non-transgenic siblings, as measured by transport of ingested beads for 2 h to the mid-posterior intestine and their complete expulsion (Fig. 5C). When larvae were sorted prior to bead feeding based upon the intensity of GFP expression, we were able to show that increased propulsion was dose dependant in that GFP expression levels, which are a surrogate for the level of CADDK51, correlated with changes in bead motility (Fig. 5D). Increased intestinal propulsion in transgenic larvae was not related to altered contractile rate (Figure S4).

In addition to the *Tg(sm22a:CADDK51-GFP)* line, we generated a second line, *Tg(sm22a:CaDMG101-GFP)* with smooth muscle expression of an inhibitory peptide derived from the h-CaD actin-binding domain.³⁷ The CaDMG101-GFP peptide also had a pronounced enhancing effect on intestinal motility similar to that of the CADDK51 peptide (Fig. 5C).

h-CaD modulates endogenous smooth muscle contraction independent of phospho-Mlc

To address the role of thin filament regulation of intestinal peristalsis independent of phospho-Mlc, we took advantage of a zebrafish mutant, *sox10^{colourless} (cls)* that lacks enteric nerves.^{39,40} In previous work, we showed that *cls* mutants have nearly undetectable levels of intestinal phospho-Mlc and phospho-h-CaD, but normal total h-CaD levels.³⁰ Surprisingly, even though *cls* larvae had little if any intestinal phospho-Mlc, they had a

significant amount of residual propulsive peristaltic activity.³⁰ This suggested that peristalsis was driven by smooth muscle contraction arising from intrinsic actomyosin interactions. Low phospho-h-CaD levels in *cls* suggested that baseline peristalsis was inhibited and might be enhanced by h-CaD knockdown or by disrupting h-CaD interaction with either myosin or actin filaments. To determine if the lack of phospho-h-CaD inhibited peristalsis in the absence of neuronal input, we repeated the h-CaD morpholino experiments in *cls* mutant larvae (Fig. 6A). In *cls* larvae injected with *Cald1a-i4e5* MO, intestinal motility was assayed at 5 dpf. As a result of reduced uptake of the beads and slower intestinal transit by *cls* mutants, larvae in this assay were exposed to the beads for approximately 6 h and assayed 20 h later. This experiment showed enhanced propulsive peristalsis in h-CaD deficient *cls* larvae (Fig. 6A). While fewer of the *Cald1a-i4e5* MO *cls* larvae expelled the beads (2%, compared to 21% of wild type larvae), many more had transported the beads to the mid-posterior intestine compared with control *cls* larvae (68% vs. 15%, respectively).

Next, we assayed peristalsis in 7 dpf and 9 dpf *cls* larvae that express the myosin-binding peptide in intestinal smooth muscle. As these larvae were older than the *Cald1a-i4e5* MO injected larvae, a 2-h feeding was sufficient, and peristalsis was assayed 20 h after feeding. This experiment showed that both transport of the fluorescent beads to the mid-posterior intestine and their expulsion was significantly greater in the transgenic *cls* larvae (Fig. 6B). The increased propulsion in transgenic *cls* larvae was not related to altered contractile rate (Fig. S4). The peristaltic defect in *cls* was not fully rescued by h-CaD disruption, as wild-type larvae at both stages completely cleared all ingested beads (data not shown). This was expected, given that the enteric nervous system is the primary stimulus for smooth muscle contraction. However, these findings do show that h-CaD-mediated inhibition of intrinsic actomyosin interactions contributes to reduced intestinal motility in the non-innervated intestine of zebrafish larvae.

DISCUSSION

Although regulation of smooth muscle contraction principally occurs at the myosin (thick) filament through phosphorylation of regulatory Mlc, a potentially important role for regulation of contraction at the actin (thin) filament has long been recognized. However, the physiologic importance of this mode of regulation has never been directly assayed *in vivo*. Here, we show that the smooth muscle actin-binding protein h-CaD regulates intestinal peristalsis in the wild type and non-innervated intestine of zebrafish larvae.

The h-CaD isoform that we identified arises from alternative splicing of a pre-mRNA transcribed from *cald1a*, one of two *cald1* paralogs we show are present in the zebrafish genome. The *cald1a* locus also encodes an l-CaD isoform expressed in intestinal smooth muscle. Predicted translation of the h-CaD protein showed high sequence homology of the actin and myosin-binding domains compared with other vertebrate h-CaD proteins. In contrast, there is less sequence homology between the various spacer domains, possibly because of its proposed structural role as a charged single α -helix.^{41,42} The *cald1b* locus encodes an l-CaD isoform expressed at low levels in intestinal smooth muscle that was previously reported to play a role in vascular development.³² Expression of a *cald1b* h-CaD ortholog was not detected. Together with functional analyses described below, these findings show that *cald1a* encodes the zebrafish ortholog of the h-CaD isoform expressed in mammalian intestinal smooth muscle. These findings also argue that *cald1a* and *cald1b* have non-overlapping functions in smooth muscle, similar to other zebrafish gene paralogs that arose from genome duplication events.

We examined the role of h-CaD in intestinal peristalsis and smooth muscle contraction using a previously described simple *in vivo* assay.³⁰ Propulsive peristalsis was increased in h-CaD

deficient wild-type larvae generated via isoform specific antisense targeting. Similarly, peristalsis was increased in larvae that express peptide transgenes designed to interfere with h-CaD binding to either the myosin or actin filament. As the rate of smooth muscle contraction was not increased in transgenic larvae, our findings argue that h-CaD normally functions to inhibit the force of smooth muscle contraction, as previously reported.²⁵ Whether h-CaD affects contraction velocity or the distance of contraction in the intestine could not be determined using our methods. Although these results are consistent with the predicted role of h-CaD in modulating smooth muscle contractile force from phosphorylated cross-bridges, to our knowledge, they are the first evidence for this phenomenon *in vivo*. h-CaD deficient mice have been generated through gene targeting; however, the effect of h-CaD disruption on intestinal motility has not been reported.²⁷ Given the similarities of intestinal anatomy and physiology in zebrafish and other vertebrates, a comparable effect of h-CaD deficiency on mammalian intestinal motility seems likely.^{28,43,44}

h-CaD function has been studied extensively in vascular (tonic) smooth muscle. Besides functioning as a brake on contraction arising from phosphorylated myosin heads, h-CaD is also thought to sustain contractile force generated by non- or de-phosphorylated myosin heads, thus accounting for force generation at low levels of phospho-Mlc. The mechanism of this aspect of h-CaD function remains unknown, but it has been postulated to occur through either physical stabilization of the actomyosin complex or the cooperative activation of nonphosphorylated and phosphorylated myosin heads.⁴⁵ This presumably takes place when h-CaD itself has been inhibited, such as occurs with phosphorylation.

Whether h-CaD functions comparably in phasic smooth muscle of the intestine and colon remains unknown. Analyses of *cls* larvae provided an opportunity to examine this question *in vivo*, as they have nearly undetectable levels of intestinal phospho-Mlc.³⁰ Surprisingly, *cls* larvae retain a significant degree of peristaltic function,³⁰ presumably from ENS-independent smooth muscle contraction, which has been observed in the embryonic mouse and zebrafish intestine.⁴⁶⁻⁴⁸ In addition to very low phospho-Mlc levels, *cls* larvae also have undetectable levels of h-CaD phosphorylated at a serine residue corresponding to a major regulatory motif of human CALD1, serine-789.³⁰ Low phospho-h-CaD to total h-CaD ratio in *cls* suggested that the basal level of smooth muscle contraction might be inhibited. Our experiments here indicate that this is indeed the case as both *Cald1a-i4e5* MO injection and transgenic expression of the CADDK51 inhibitory peptide enhanced propulsive peristalsis in *cls*. Although it is difficult to make mechanistic inferences regarding h-CaD function from this study, our findings are consistent with the idea that h-CaD restricts binding of non-phosphorylated myosin heads to actin (Fig. 1C), as h-CaD knockdown enhanced peristalsis in *cls* mutants that have low levels of p-Mlc. The knockdown experiments in wild-type larvae (with high levels of p-Mlc) argue that h-CaD restricts binding of phosphorylated myosin heads to their preferred site on the actin filament (Fig. 1A) and that this restriction is relieved in the absence of h-CaD.

h-CaD is widely expressed in human smooth muscle and has been used extensively as a tumor marker.^{49,50} Its best characterized physiologic role is during pregnancy when total h-CaD levels increase,⁵¹ thus inhibiting premature stretch induced uterine contraction.⁵² At the time of delivery, phospho-h-CaD levels increase dramatically. This is thought to enhance the force of uterine contractions. A role for premature phosphorylation of h-CaD in a rodent model of preterm labor has also been reported.⁵³

Relatively little is known about the regulation or function of h-CaD in the human intestine. In rabbit colonic smooth muscle, h-CaD serine-789 is phosphorylated upon exposure to the enteric neurotransmitter acetylcholine, which also drives phosphorylation of Mlc.⁵⁴ This argues that h-CaD's normal function is to enhance ENS-mediated smooth muscle

contraction. Furthermore, it suggests that in the setting of ENS dysfunction, levels of both phospho-Mlc and phospho-CaD are reduced, as occurs in zebrafish *cls* mutants. Surprisingly, *cls* larvae retain peristaltic activity, even though they have few, if any enteric nerves. This is likely a function of smooth muscle physiology and intestinal anatomy at larval stages. By contrast, a comparable level of ENS disruption in the adult mammalian intestine, which can be modeled by conditional *Mlck* disruption in mice,⁵⁵ is predicted to cause irreversible intestinal motility defects. Hypomotility caused by a more modest reduction in ENS function, could conceivably be enhanced by modulation of h-CaD. Targeting h-CaD could therefore be a therapeutic strategy to treat hypomotility caused by enteric neuropathies and related disorders associated with reduced phospho-Mlc, such as is likely to occur with aging and disorders affecting Cajal pacemaker cells.^{56,57}

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

h-CaD	high molecular weight Caldesmon isoform
l-CaD	low molecular weight Caldesmon isoform
ENS	enteric nervous system
Mlc	myosin light chain
Mlck	myosin light chain kinase
dpf	days post fertilization

REFERENCES

1. Kamm KE, Stull JT. The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu Rev Pharmacol Toxicol.* 1985; 25:593–620. [PubMed: 2988424]
2. Murphy RA. Contraction in smooth muscle cells. *Annu Rev Physiol.* 1989; 51:275–83. [PubMed: 2653182]
3. Siegman MJ, Butler TM, Mooers SU, Michalek A. Ca²⁺ can affect V_{max} without changes in myosin light chain phosphorylation in smooth muscle. *Pflugers Arch.* 1984; 401:385–90. [PubMed: 6483580]
4. Gerthoffer WT. Dissociation of myosin phosphorylation and active tension during muscarinic stimulation of tracheal smooth muscle. *J Pharmacol Exp Ther.* 1987; 240:8–15. [PubMed: 3100773]
5. Haeberle JR, Hott JW, Hathaway DR. Regulation of isometric force and isotonic shortening velocity by phosphorylation of the 20,000 dalton myosin light chain of rat uterine smooth muscle. *Pflugers Arch.* 1985; 403:215–9. [PubMed: 3982971]
6. Moreland S, Moreland RS. Effects of dihydropyridines on stress, myosin phosphorylation, and V₀ in smooth muscle. *Am J Physiol.* 1987; 252:H1049–58. [PubMed: 2438947]
7. Gusev NB. Some properties of caldesmon and calponin and the participation of these proteins in regulation of smooth muscle contraction and cytoskeleton formation. *Biochemistry.* 2001; 66:1112–21. [PubMed: 11736632]

8. Sobue K, Muramoto Y, Fujita M, Kakiuchi S. Purification of a calmodulin-binding protein from chicken gizzard that interacts with F-actin. *Proc Natl Acad Sci USA*. 1981; 78:5652–5. [PubMed: 6946503]
9. Kordowska J, Huang R, Wang CL. Phosphorylation of caldesmon during smooth muscle contraction and cell migration or proliferation. *J Biomed Sci*. 2006; 13:159–72. [PubMed: 16453176]
10. Helfman DM, Levy ET, Berthier C, et al. Caldesmon inhibits nonmuscle cell contractility and interferes with the formation of focal adhesions. *Mol Biol Cell*. 1999; 10:3097–112. [PubMed: 10512853]
11. Wang CL, Chalovich JM, Graceffa P, Lu RC, Mabuchi K, Stafford WF. A long helix from the central region of smooth muscle caldesmon. *J Biol Chem*. 1991; 266:13958–63. [PubMed: 1856225]
12. Horiuchi KY, Miyata H, Chacko S. Modulation of smooth muscle actomyosin ATPase by thin filament associated proteins. *Biochem Biophys Res Commun*. 1986; 136:962–8. [PubMed: 2941015]
13. Earley JJ, Su X, Moreland RS. Caldesmon inhibits active crossbridges in unstimulated vascular smooth muscle: an antisense oligodeoxynucleotide approach. *Circ Res*. 1998; 83:661–7. [PubMed: 9742062]
14. Szpacenko A, Wagner J, Dabrowska R, Ruegg JC. Caldesmon-induced inhibition of ATPase activity of actomyosin and contraction of skinned fibres of chicken gizzard smooth muscle. *FEBS Lett*. 1985; 192:9–12. [PubMed: 2932346]
15. Ansari S, Alahyan M, Marston SB, El-Mezgueldi M. Role of caldesmon in the Ca²⁺ regulation of smooth muscle thin filaments: evidence for a cooperative switching mechanism. *J Biol Chem*. 2008; 283:47–56. [PubMed: 17933868]
16. Wang CL. Caldesmon and smooth-muscle regulation. *Cell Biochem Biophys*. 2001; 35:275–88. [PubMed: 11894847]
17. Smith CW, Marston SB. Disassembly and reconstitution of the Ca²⁺-sensitive thin filaments of vascular smooth muscle. *FEBS Lett*. 1985; 184:115–9. [PubMed: 3987897]
18. Ikebe M, Reardon S. Phosphorylation of smooth muscle caldesmon by calmodulin-dependent protein kinase II. Identification of the phosphorylation sites. *J Biol Chem*. 1990; 265:17607–12. [PubMed: 2170388]
19. Childs TJ, Watson MH, Sanghera JS, Campbell DL, Pelech SL, Mak AS. Phosphorylation of smooth muscle caldesmon by mitogen-activated protein (MAP) kinase and expression of MAP kinase in differentiated smooth muscle cells. *J Biol Chem*. 1992; 267:22853–9. [PubMed: 1331069]
20. Katsuyama H, Wang CL, Morgan KG. Regulation of vascular smooth muscle tone by caldesmon. *J Biol Chem*. 1992; 267:14555–8. [PubMed: 1386078]
21. Horiuchi KY, Chacko S. Caldesmon inhibits the cooperative turning-on of the smooth muscle heavy meromyosin by tropomyosin-actin. *Biochemistry*. 1989; 28:9111–6. [PubMed: 2532547]
22. Lu C, Liu Y, Tang X, Ye H, Zhu D. Role of 15-hydroxyeicosatetraenoic acid in phosphorylation of ERK1/2 and caldesmon in pulmonary arterial smooth muscle cells. *Can J Physiol Pharmacol*. 2006; 84:1061–9. [PubMed: 17218971]
23. Zhang H, Zhang L. Regulation of alpha1-adrenoceptor-mediated contractions of the uterine artery by protein kinase C: role of the thick- and thin-filament regulatory pathways. *J Pharmacol Exp Ther*. 2007; 322:1253–60. [PubMed: 17562849]
24. Chacko S, Chang S, Hypolite J, Disanto M, Wein A. Alteration of contractile and regulatory proteins following partial bladder outlet obstruction. *Scand J Urol Nephrol Suppl*. 2004; 215:26–36. [PubMed: 15545194]
25. Smolock EM, Trapanese DM, Chang S, Wang T, Titchenell P, Moreland RS. siRNA-mediated knockdown of h-caldesmon in vascular smooth muscle. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1930–9. [PubMed: 19767533]
26. Gerthoffer WT, Pohl J. Caldesmon and calponin phosphorylation in regulation of smooth muscle contraction. *Can J Physiol Pharmacol*. 1994; 72:1410–4. [PubMed: 7767886]
27. Guo H, Wang CL. Specific disruption of smooth muscle caldesmon expression in mice. *Biochem Biophys Res Commun*. 2005; 330:1132–7. [PubMed: 15823561]

28. Wallace KN, Akhter S, Smith EM, Lorent K, Pack M. Intestinal growth and differentiation in zebrafish. *Mech Dev.* 2005; 122:157–73. [PubMed: 15652704]
29. Lee YH, Gallant C, Guo H, Li Y, Wang CA, Morgan KG. Regulation of vascular smooth muscle tone by N-terminal region of caldesmon. Possible role of tethering actin to myosin. *J Biol Chem.* 2000; 275:3213–20. [PubMed: 10652307]
30. Davuluri G, Seiler C, Abrams J, Soriano AJ, Pack M. Differential effects of thin and thick filament disruption on zebrafish smooth muscle regulatory proteins. *Neurogastroenterol Motil.* 2010; 22:1100–e1285. [PubMed: 20591105]
31. Amores A, Force A, Yan YL, et al. Zebrafish hox clusters and vertebrate genome evolution. *Science.* 1998; 282:1711–4. [PubMed: 9831563]
32. Zheng PP, Severijnen LA, van der Weiden M, Willemsen R, Kros JM. A crucial role of caldesmon in vascular development in vivo. *Cardiovasc Res.* 2009; 81:362–9. [PubMed: 18980955]
33. Bryan J. Caldesmon, acidic amino acids and molecular weight determinations. *J Muscle Res Cell Motil.* 1989; 10:95–6. [PubMed: 2668328]
34. Seiler C, Abrams J, Pack M. Characterization of zebrafish intestinal smooth muscle development using a novel sm22alpha-b promoter. *Dev Dyn.* 2010; 239:2806–12. [PubMed: 20882680]
35. Ikebe M, Reardon S. Binding of caldesmon to smooth muscle myosin. *J Biol Chem.* 1988; 263:3055–8. [PubMed: 3257755]
36. Wang Z, Jiang H, Yang ZQ, Chacko S. Both N-terminal myosin-binding and C-terminal actin-binding sites on smooth muscle caldesmon are required for caldesmon-mediated inhibition of actin filament velocity. *Proc Natl Acad Sci USA.* 1997; 94:11899–904. [PubMed: 9342334]
37. Zhan QQ, Wong SS, Wang CL. A calmodulin-binding peptide of caldesmon. *J Biol Chem.* 1991; 266:21810–4. [PubMed: 1939204]
38. Provost E, Rhee J, Leach SD. Viral 2A peptides allow expression of multiple proteins from a single ORF in transgenic zebrafish embryos. *Genesis.* 2007; 45:625–9. [PubMed: 17941043]
39. Kelsh RN, Eisen JS. The zebrafish colourless gene regulates development of non-ectomesenchymal neural crest derivatives. *Development.* 2000; 127:515–25. [PubMed: 10631172]
40. Dutton KA, Pauliny A, Lopes SS, et al. Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development.* 2001; 128:4113–25. [PubMed: 11684650]
41. Mabuchi K, Wang CL. Electron microscopic studies of chicken gizzard caldesmon and its complex with calmodulin. *J Muscle Res Cell Motil.* 1991; 12:145–51. [PubMed: 2061408]
42. Suveges D, Gaspari Z, Toth G, Nyi-tray L. Charged single alpha-helix: a versatile protein structural motif. *Proteins.* 2009; 74:905–16. [PubMed: 18712826]
43. Rich A, Leddon SA, Hess SL, et al. Kit-like immunoreactivity in the zebrafish gastrointestinal tract reveals putative ICC. *Dev Dyn.* 2007; 236:903–11. [PubMed: 17295318]
44. Shepherd I, Eisen J. Development of the zebrafish enteric nervous system. *Methods Cell Biol.* 2011; 101:143–60. [PubMed: 21550442]
45. Albrecht K, Schneider A, Liebetrau C, Ruegg JC, Pfitzer G. Exogenous caldesmon promotes relaxation of guinea-pig skinned taenia coli smooth muscles: inhibition of cooperative reattachment of latch bridges? *Pflugers Arch.* 1997; 434:534–42. [PubMed: 9242716]
46. Roberts RR, Murphy JF, Young HM, Bornstein JC. Development of colonic motility in the neonatal mouse—studies using spatiotemporal maps. *Am J Physiol Gastrointest Liver Physiol.* 2007; 292:G930–8. [PubMed: 17158255]
47. Roberts RR, Ellis M, Gwynne RM, et al. The first intestinal motility patterns in fetal mice are not mediated by neurons or interstitial cells of Cajal. *J Physiol.* 2010; 588:1153–69. [PubMed: 20142273]
48. Holmberg A, Olsson C, Hennig GW. TTX-sensitive and TTX-insensitive control of spontaneous gut motility in the developing zebrafish (*Danio rerio*) larvae. *J Exp Biol.* 2007; 210:1084–91. [PubMed: 17337720]
49. Miettinen M, Lasota J. Gastrointestinal stromal tumors: review on morphology, molecular pathology, prognosis, and differential diagnosis. *Arch Pathol Lab Med.* 2006; 130:1466–78. [PubMed: 17090188]

50. McCluggage WG. A critical appraisal of the value of immunohistochemistry in diagnosis of uterine neoplasms. *Adv Anat Pathol*. 2004; 11:162–71. [PubMed: 15096730]
51. Word RA, Stull JT, Casey ML, Kamm KE. Contractile elements and myosin light chain phosphorylation in myometrial tissue from nonpregnant and pregnant women. *J Clin Invest*. 1993; 92:29–37. [PubMed: 8392087]
52. Li Y, Reznichenko M, Tribe RM, et al. Stretch activates human myometrium via ERK, caldesmon and focal adhesion signaling. *PLoS ONE*. 2009; 4:e7489. [PubMed: 19834610]
53. Li Y, Je HD, Malek S, Morgan KG. Role of ERK1/2 in uterine contractility and preterm labor in rats. *Am J Physiol Regul Integr Comp Physiol*. 2004; 287:R328–35. [PubMed: 15072963]
54. Somara S, Bitar KN. Phosphorylated HSP27 modulates the association of phosphorylated caldesmon with tropomyosin in colonic smooth muscle. *Am J Physiol Gastrointest Liver Physiol*. 2006; 291:G630–9. [PubMed: 16627824]
55. He WQ, Peng YJ, Zhang WC, et al. Myosin light chain kinase is central to smooth muscle contraction and required for gastrointestinal motility in mice. *Gastroenterology*. 2008; 135:610–20. [PubMed: 18586037]
56. Somara S, Gilmont RR, Martens JR, Bitar KN. Ectopic expression of caveolin-1 restores physiological contractile response of aged colonic smooth muscle. *Am J Physiol Gastrointest Liver Physiol*. 2007; 293:G240–9. [PubMed: 17431219]
57. Farrugia G. Interstitial cells of Cajal in health and disease. *Neurogastroenterol Motil*. 2008; 20:54–63. [PubMed: 18402642]

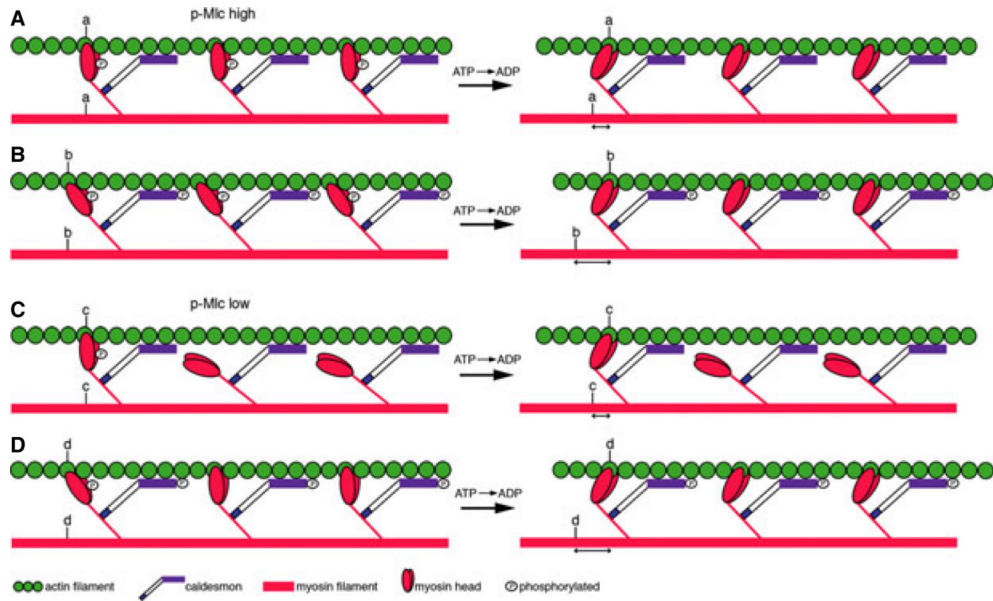


Figure 1. Simplified model of h-CaD function. Smooth muscle contraction depicted by sliding of actin (green) on myosin (red) filaments, following ATP hydrolysis (derived from previously described model¹⁶). (A) In smooth muscle with high levels of phospho-Mlc (p-Mlc), non-phosphorylated h-CaD restricts binding of myosin heads to the actin filament such that force generation is inhibited. (B) When h-CaD is phosphorylated, the myosin heads are able to bind the actin filament in a manner that enhances actomyosin interaction (b). This leads to increased force generation (distance b > distance a). (C) In smooth muscle with low levels of p-Mlc, non-phosphorylated h-CaD prevents binding of non-phosphorylated myosin heads to actin. (D) phospho-h-CaD promotes binding of the non-phosphorylated myosin heads to actin, thereby enhancing contraction; (distance d > distance c). Distances a, b and c, d are not drawn to scale.

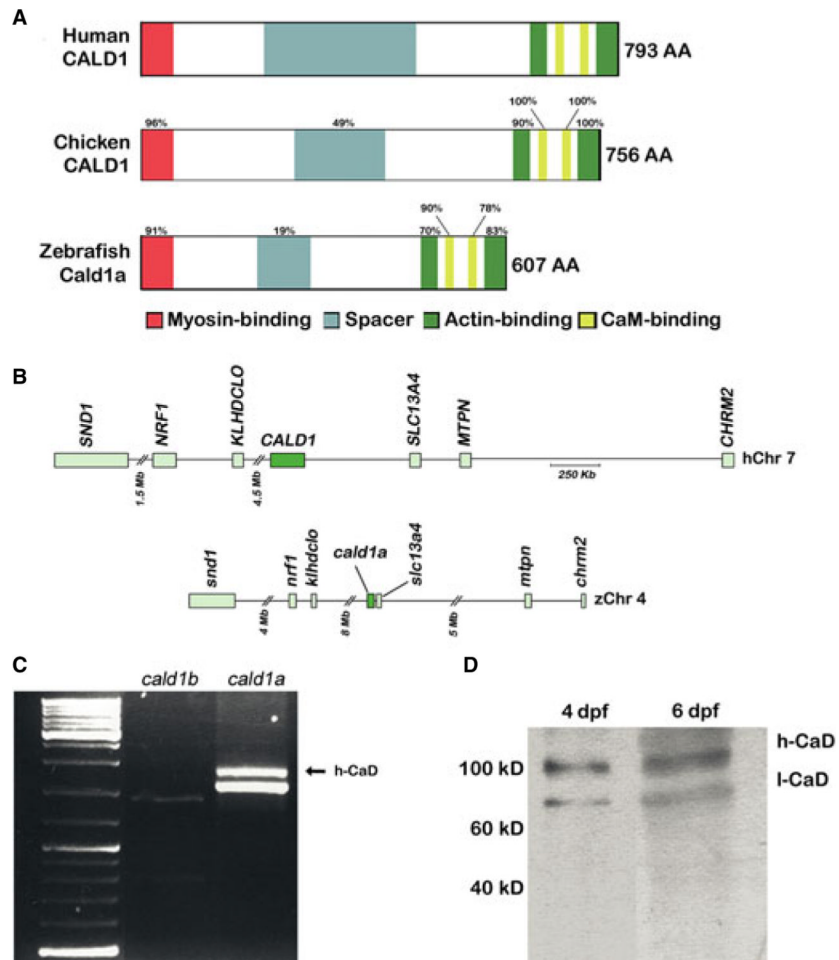


Figure 2. Zebrafish smooth muscle Caldesmon (h-CaD). (A) Schematic representation of the human, chicken and zebrafish h-CaD orthologs. Conserved protein domains and percent amino acid homology are indicated. (B) Conserved syntenic relationships surrounding the human *CALD1* locus on chromosome 7 and the zebrafish *cald1a* locus on chromosome 4. (C) RT-PCR showing amplification of the full length cDNA corresponding to the high and low molecular weight zebrafish *cald1a* isoforms from intestinal cDNA. A correctly sized transcript for the predicted low molecular weight *cald1b* isoform is also detected. (D) Western blot showing intestinal levels of CaD isoforms. Molecular weight standards indicated. Mb, megabase; Kb, kilobase; dpf, days post fertilization.

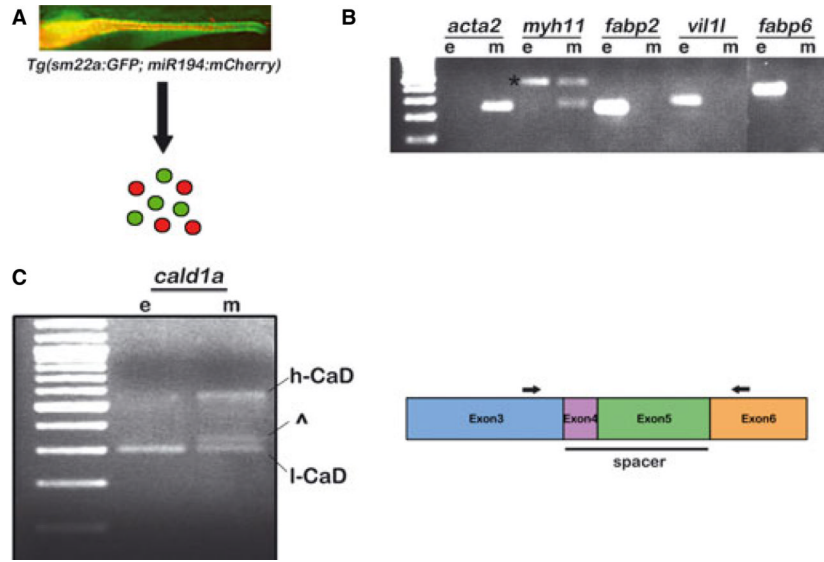


Figure 3. Expression of the zebrafish h-CaD ortholog in intestinal smooth muscle. (A) Scheme to isolate intestinal smooth muscle (green) and epithelial (red) cells from *Tg(sm22a:GFP; miR194:mCherry)* larvae. Fluorescent image of the intestine of a 5 dpf bigenic larva is shown. Smooth muscle of the anterior intestine is not in plane of focus. (B) RT-PCR amplification showing expression of intestinal smooth muscle and epithelial markers in sorted cells. The *myh11* primers amplify a band (*) from contaminating epithelial and smooth muscle genomic DNA (confirmed by sequencing). (C) Expression of the high molecular weight *cald1a* transcript is restricted to smooth muscle. In some experiments, an additional band was amplified from smooth muscle that migrated near the low molecular weight *cald1* transcript (^); however, this was detected in only a minority of experiments. Schematic indicates the location of PCR primers (arrows) in exon 3 and exon 6 that were used to amplify the *cald1a* isoforms. e, epithelial; m, smooth muscle.

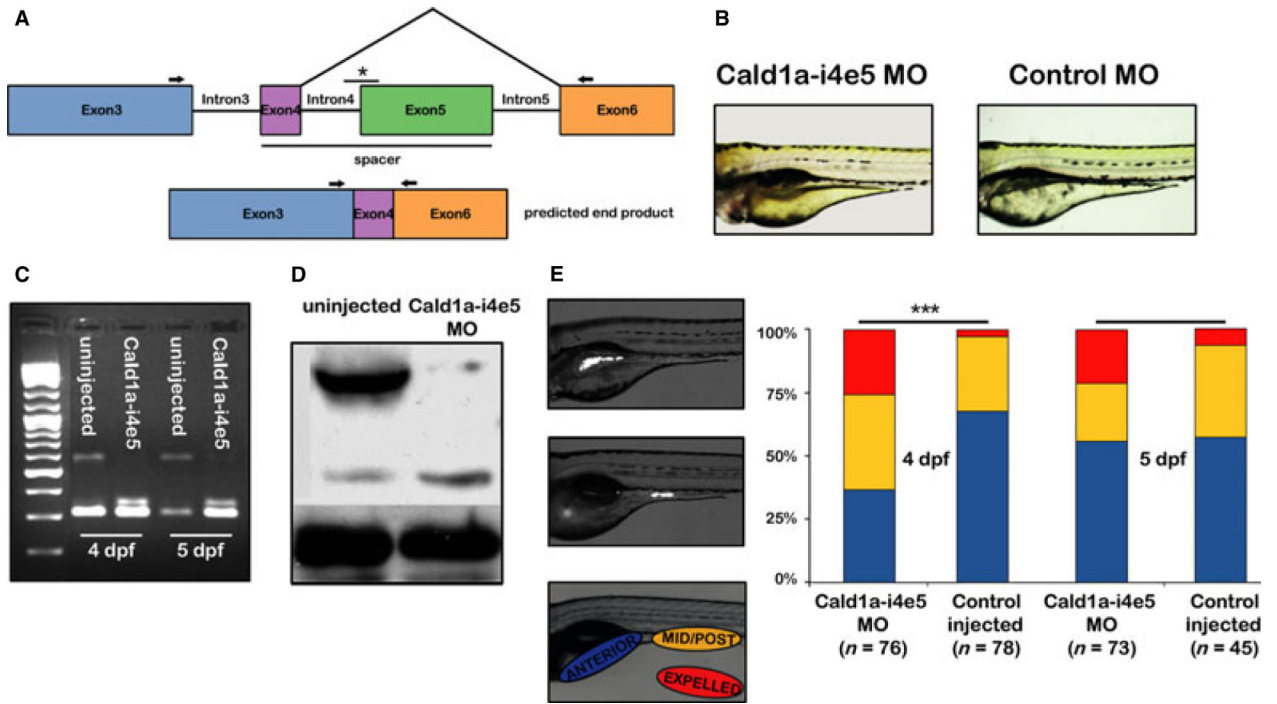


Figure 4.

h-CaD deficiency enhances intestinal peristalsis in zebrafish larvae. (A) Schematic depicting isoform specific targeting of the high molecular weight *cald1a* transcript by a splice blocking morpholino (Cald1a-i4e5 MO). The exon 5 splice acceptor is targeted (*). (B) Normal morphology of 4 dpf larvae injected with Cald1a-i4e5 MO and control morpholinos. (C) RT-PCR of intestinal cDNA from Cald1a-i4e5 MO larvae using exon 3 and exon 6 primers (arrows in panel 3A) shows markedly reduced expression of the high molecular weight *cald1a* transcript. (D) Western blot using intestinal protein from Cald1a-i4e5 MO larvae shows reduced levels of h-CaD with slightly increased l-CaD levels. Increased protein corresponding to l-CaD likely reflects additional protein translated from modified transcript only 12 amino acids larger than l-CaD. (E) Images of live 5 dpf larvae that ingested fluorescent microspheres located in anterior and mid-posterior intestine, respectively. Color scheme in bar graph depicts bead location indicated in lateral image of larva (lower panel). Cald1a-i4e5 MO larvae show increased propulsive peristalsis at 4dpf (chi-squared test, *** $P < 0.001$). 5dpf P -value = 0.08, likely due to transient effect of morpholino knockdown.

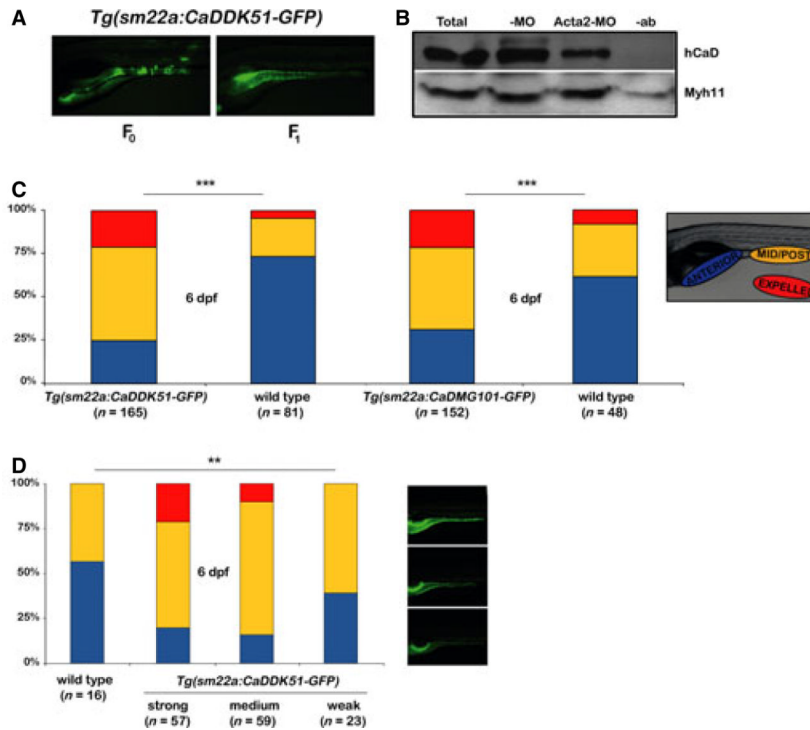


Figure 5. Expression of peptides blocking h-CaD interaction at myosin and actin filaments increases intestinal propulsive peristalsis. (A) Lateral images of live 5 dpf F₀ and F₁ *Tg(sm22a:CaDDK51-GFP)* larvae showing mosaic and widespread smooth muscle GFP expression, respectively. (B) IP-Western blot confirms that CaDDK51 myosin-binding domain peptide blocks interaction of h-CaD with Myh11. Total intestinal protein was IP'ed with Myh11 antibody and blotted as indicated. (C) Bar graph shows increased propulsive peristalsis in unsorted *Tg(sm22a:CaDDK51-GFP)* larvae and *Tg(sm22a:CaDMG101-GFP)* larvae, in which peptides block h-CaD interaction with myosin and actin, respectively (chi-squared test, ****P* < 001). (D) Enhanced peristalsis in *Tg(sm22a:CaDDK51-GFP)* larvae correlates with the level of transgene expression (determined by GFP fluorescence). Images show larvae with strong, medium and weak GFP expression (Fisher's exact test, ***P* < 05). -MO – control larvae; Acta2-MO – larvae injected with smooth muscle actin morpholino; -ab – control IP without antibody; Myh11 – smooth muscle myosin.

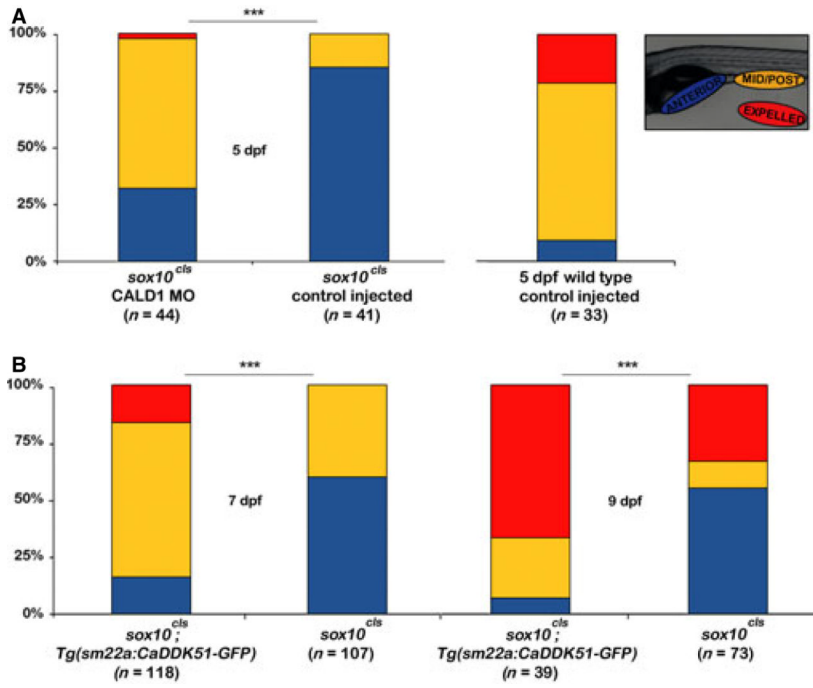


Figure 6. Intestinal peristalsis is increased in h-CaD deficient colourless larvae. (A) Bar graph shows partial rescue of intestinal peristalsis in h-CaD deficient 5 dpf *cls* larvae. (B) *Tg (sm22a: CaDDK51 -GFP) cls* larvae show partial rescue of propulsive peristalsis at 7 dpf and 9 dpf (chi-squared test, *** $P < 001$).