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Analysis Of A Zebrafish Behavioral Mutant Reveals A Dominant Mutation In *atp2a1/SERCA1*

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Abstract

Zebrafish embryos demonstrate robust swimming behavior, which consists of smooth, alternating body bends. In contrast, several motility mutants have been identified that perform sustained, bilateral trunk muscle contractions which result in abnormal body shortening. Unlike most of these mutants, *accordion* (*acc*)^{*dtas*} demonstrates a semidominant effect: Heterozygotes exhibit a distinct but less severe phenotype than homozygotes. Using molecular-genetic mapping and candidate gene analysis, we determined that *acc*^{*dtas*} mutants harbor a novel mutation in *atp2a1*, which encodes SERCA1, a calcium pump important for muscle relaxation. Previous studies have shown that eight other *acc* alleles compromise SERCA1 function, but these alleles were all reported to be recessive. Quantitative behavioral assays, complementation testing, and analysis of molecular models all indicate that the *acc*^{*dtas*} mutation diminishes SERCA1 function to a greater degree than other *acc* alleles through either haploinsufficient or dominant-negative molecular mechanisms. Since mutation of human *ATP2A1* results in Brody disease, an exercise induced impairment of muscle relaxation, *acc*^{*dtas*} mutants may provide a particularly sensitive model of this disorder.

Keywords

Danio rerio; zebrafish; mutant; behavior; muscle; Brody disease

Zebrafish embryos perform an initial sequence of three motor behaviors as they develop. Initially, at about 17 hours postfertilization (hpf), embryos demonstrate spontaneous, alternating tail coils. Around 21 hpf, embryos begin to respond to touch by performing more rapid tail coils. Finally, embryos begin to swim at approximately 27 hpf, and react to touch by moving at least one body length away from the touch stimulus (Downes and Granato, 2006; Saint-Amant and Drapeau, 1998). Granato *et al.* utilized this series of robust behaviors as the basis for extensive mutagenesis screens to identify mutants with specific deficits in embryonic motility (Granato *et al.*, 1996). Many mutants demonstrated similar behavioral deficits, which was used as criteria to group them into several different phenotypic classes.

All members of the accordion class of motility mutants exhibit similar, characteristic behavior. Beginning at the onset of the touch response, accordion class mutants perform bilateral trunk muscle contractions, which cause the embryo to contract along the rostral-caudal axis reminiscent of an accordion. The genes have been identified for several

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accordion class mutants and, despite the similar behavior of the mutants, they encode proteins essential for diverse aspects of spinal cord, neuromuscular synapse or muscle function (Downes and Granato, 2004; Gleason *et al.*, 2004; Hirata *et al.*, 2005; Hirata *et al.*, 2004; Lefebvre *et al.*, 2004; Wang *et al.*, 2008).

A single allele of *dta5* was isolated in a previous genetic screen for dominant mutants that exhibit abnormal locomotor behavior (van Eeden *et al.*, 1999). As a first step to determine the molecular and cellular defects in *dta5* mutants, we analyzed their locomotive behavior over the course of development using a high-speed video camera. Homozygous mutant embryos perform abnormal behavior that can first be distinguished from wild-type embryos around 24 hours postfertilization (hpf). At this time point, wild-type embryos respond to touch with smooth, alternating tail coils (Supporting information: Movie1). In contrast, *dta5* homozygotes appear to contract their trunk muscles bilaterally, which results in prolonged compression along the rostral-caudal axis and an aberrant dorsal bend (Supporting information: Movie2). *dta5* heterozygotes demonstrate an intermediate phenotype consisting of slower or incomplete tail coils. The heterozygous phenotype is variable and challenging to quantify at 24hpf, but analysis of a population of embryos obtained from crossing *dta5* heterozygotes revealed that 61.3% exhibit abnormal behavior at this stage of development (n=186; data not shown). According to Mendelian ratios, about 25% of the embryos would be predicted to show abnormal behavior if *dta5* were a recessive allele. Therefore, this observation shows that *dta5* homozygotes and some heterozygotes can be identified at 24hpf and it demonstrates the dominant mode of inheritance of *dta5*.

At 48hpf, wild-type larvae exhibit robust swimming behavior, but both *dta5* heterozygotes and homozygotes continue to display abnormal motor behavior. Kinematic analysis illustrates that wild-type larvae respond to touch with a rapid burst of swimming, consisting of fast, alternating body bends (Figure 1a, d; Supporting information: Movie3). *dta5* homozygotes do not swim in response to touch stimuli. They typically perform a small amplitude body bend towards one side and appear stiff, likely due to bilateral trunk muscle contractions (Figure 1c, f; Supporting information: Movie4). The rostral-caudal compression and dorsal bend observed at early ages are less pronounced at 48hpf, presumably because of the mechanical resistance provided by the increased stiffness of the notochord. *dta5* heterozygotes display an intermediate phenotype (Figure 1b, e). They are able to swim; however, their swimming behavior consists of slow, small-amplitude body bends. As a result, *dta5* heterozygotes swim much more slowly than wild-type embryos (compare Figure 1a and 1b; Supporting information: Movie5).

Since we observed a clear correlation between swimming speed and genotype, we developed a behavioral assay to measure the amount of time it takes larval zebrafish to swim a 4mm distance. Using this time-trial assay, we found that a population of wild-type larvae swim with an average time of 137ms at 48hpf (Figure 2a). Wild-type larvae swim faster over the course of development, with average times of 76ms at 96hpf and 43ms at 144hpf (Figure 2b, c). A population of larvae obtained from crossing *dta5* heterozygotes showed a range of swimming times at 48hpf. We expected to observe a Mendelian distribution of 25% wild-type with normal swimming times, 50% heterozygotes with abnormally slow swimming times, and 25% homozygotes that are unable to swim. Instead, we noted that about 36% exhibit wild-type like swimming times, 34% demonstrate abnormally slow swimming times, defined as more than 500ms, and 30% are unable to swim (Figure 2d). This suggests that *dta5* heterozygotes exhibit a wide range of swimming phenotypes at 48hpf, with some indistinguishable from wild-type or *dta5* homozygotes. Regardless of genotype, nearly all larvae obtained from crossing *dta5* heterozygotes acquire the ability to swim (Figure 2e, f). While most *dta5* homozygotes can swim by 144hpf, their swimming is not normal. Abnormalities in tail-flip frequency and left-right alternation were observed (data not

shown). These larvae fail to inflate the swim bladder and soon die, likely due to an inability to feed. We did not observe any differences in swimming behavior between *dta5* heterozygotes and wild-type larvae at 144hpf, and *dta5* heterozygotes are viable to adulthood.

To determine the molecular identity of *dta5*, we mapped its genomic location using a three-generation map cross panel. Genomic DNA from pooled and individual wild-type and homozygous mutant embryos was screened with a panel of simple sequence length polymorphism (SSLP) markers. We found that *dta5* maps to chromosome 3 near markers z59380 and z68358 (both 0 recombinants in 184 meioses, Figure 3a). Genome database analysis indicated that *atp2a1* lies within this vicinity. *atp2a1* encodes Sarco(endo)plasmic Reticulum Ca^{2+} ATPase 1 (SERCA1), a calcium ATPase that plays an important role in muscle relaxation. SERCA1 transports calcium from the cytosol into the sarco(endo)plasmic reticulum of muscle, which allows actin and myosin to disassociate, resulting in muscle relaxation. Previous studies described the zebrafish *accordion* (*acc*) mutants, in the *accordion* class, which contain mutations in *atp2a1* and exhibit a behavioral phenotype similar to *dta5* (Gleason *et al.*, 2004; Hirata *et al.*, 2004).

Since *atp2a1* was a promising candidate gene for *dta5*, we cloned and sequenced *atp2a1* transcripts from heterozygous and homozygous larvae. We found a single point mutation that segregates along with the phenotype and results in the substitution of Gly598 for a Val (Figure 3b). This residue is precisely conserved among each of the three SERCA-encoding genes in vertebrates and single orthologs in *C. Elegans* and *Drosophila*, which strongly argues that Gly598 is crucial for SERCA function (Figure 3c).

Structural studies of mammalian SERCA molecules also suggest that Gly598 is important for SERCA function. Mammalian SERCA1 has been examined extensively through X-ray crystallography and site-directed mutagenesis (Toyoshima and Inesi, 2004). Based upon these studies, Gly598 is located in the ATP binding domain near where it connects to the phosphorylation domain (Figure 3d). Although this residue has not been the target of site-directed mutagenesis, mutation of nearby Asp601 and Pro603 has been shown to severely compromise Ca^{2+} transport activity (Clarke *et al.*, 1990). These residues are thought to be important in guiding the transition of SERCA from the E_1P to the E_2P state, which is important in translocating Ca^{2+} across the membrane. The substitution of Gly598 with the slightly larger Val likely disrupts the function of this critical region and greatly diminishes or abolishes SERCA1 activity. Based upon our mapping data, the nature of the *dta5* mutation, and similarity to the *acc* mutant phenotype, we propose that *dta5* is an allele of *acc*, and hereafter refer to *dta5* as *acc^{dta5}*.

Although eight other *acc* mutant alleles have been identified, each is believed to demonstrate a recessive mode of inheritance and no heterozygous phenotype has been reported (Gleason *et al.*, 2004; Granato *et al.*, 1996; Hirata *et al.*, 2004). To confirm these observations and compare one of these alleles to *acc^{dta5}*, *acc^{tq206}*, the strongest previously reported allele, was used for time-trial assays (Figure 4). A population of larvae obtained from crossing *acc^{dta5}* heterozygotes again show a range of swimming times and demonstrate the consistent results obtained using this assay (Figure 4b). 39% exhibit wild-type like swimming, 28% perform slower swimming, and 33% do not swim at all. The difference in average swim times between the populations of *acc^{dta5}* larvae shown in Figure 2d and 4b is not statistically significant ($t(92) = 0.02$, $p = .49$). Since *acc^{tq206}* were reported as recessive, we expected to observe a roughly Mendelian distribution of 75% with a wild-type phenotype and 25% that do not swim. Instead we observe that 62% perform wild-type like swimming, 5% demonstrate slower swimming, and 34% do not swim, which suggests that *acc^{tq206}* may not be fully recessive. Since *acc^{dta5}* demonstrates a semi-dominant mode of inheritance, we

expected that crosses between *acc^{dta5}* heterozygotes and wild-type fish would yield a population of larvae in which some swim like wild-type and others swim slowly. We observe that 59% swim with wild-type swim speeds, 38% swim slowly, and 3% do not swim at all, which provides additional evidence that *acc^{dta5}* is semi-dominant and that *acc^{dta5}* heterozygotes demonstrate a range of swimming phenotypes (Figure 4d). If *acc^{tq206}* is completely recessive, we would predict that crosses between *acc^{tq206}* heterozygotes and wild-type would yield a population of larvae in which all perform wild-type swimming. We find that 90% perform wild-type swimming, but 9% swim slowly and 1% do not swim. Moreover, the average swim time is significantly slower than wild-type ($t(158) = 10.08, p < .01$) (Figure 4e). These results further suggest that *acc^{tq206}* is not completely recessive. Lastly, we performed complementation testing between *acc^{dta5}* and *acc^{tq206}* to confirm that they are allelic (Figure 4f). We observe the expected failure to complement. 52% exhibit wild-type swimming speeds, 17% perform abnormally slow swimming, and 30% do not swim. Altogether, these data indicate that *acc^{dta5}* is semi-dominant, *acc^{tq206}* has a subtle semi-dominant effect, *acc^{dta5}* is a stronger allele than *acc^{tq206}*, and both alleles disrupt SERCA1 function.

In this study, we used novel quantification of swimming behavior, molecular-genetic mapping and candidate gene analysis to characterize the *acc^{dta5}* mutant over the course of development and reveal that it harbors a semi-dominant mutation in *atp2a1*. *atp2a1* is expressed in muscle (see Supporting Information: Figure 5) and it is important for muscle relaxation, so *acc^{dta5}* mutants develop abnormal swimming due to a reduced ability to relax axial muscle. Intriguingly, previous studies have indicated that eight *acc* alleles disrupt *atp2a1* and demonstrate a recessive mode of inheritance (Gleason *et al.*, 2004; Granato *et al.*, 1996; Hirata *et al.*, 2004). The sensitivity of our time-trial assay allowed us to uncover that the *acc^{tq206}* allele has a weak semi-dominant effect, with some heterozygotes having a subtle motility phenotype. This assay also shows that the *acc^{tq206}* allele is not as strong as *acc^{dta5}*, which is the strongest allele reported to date.

It is not entirely clear why the *acc^{dta5}* allele is stronger than the other *acc* alleles, but one factor is that a different portion of the SERCA1 protein is disrupted in *acc^{dta5}* mutants. The precise mutation has been determined for three *acc* alleles: *acc^{mi25i}* Ile97 is mutated to Asn, *acc^{tq206}* Ser766 is mutated to Phe, and *acc^{mi289a}* Thr848 is mutated to Ile (see Figure 3). Although these mutations are distributed across the SERCA1 protein, each is in a transmembrane domain and results in altered hydrophobicity. *acc^{dta5}*, which mutates Gly598 to Val, is the only *acc* allele identified that disrupts the SERCA1 ATP binding domain.

acc^{dta5} could result in a dominant mode of inheritance through either haploinsufficient or dominant-negative molecular mechanisms. According to a haploinsufficient model, two functional copies of *atp2a1* are required in the zebrafish embryo. *acc^{dta5}* would be a semi-dominant mutation because it diminishes SERCA1 function to a greater degree than other *acc* mutations, which all have reduced, but not abolished, SERCA1 function. According to a dominant-negative model, *acc^{dta5}* SERCA1 protein interferes with wild-type SERCA1 function. This model implies that SERCA1 requires assembly of macromolecular complexes to function. SERCA1 is generally thought to operate as a monomer but this view is being increasingly challenged by reports that SERCA1 forms oligomers (Maguire and Ohlendieck, 1996; Mahaney *et al.*, 2008). Additional analysis of the *acc^{dta5}* mutation could yield new insight into the molecular mechanisms of SERCA1 function.

We observed that both *acc^{dta5}* homozygotes and heterozygotes demonstrated a marked improvement in swimming performance over the course of development (see Figure 2). The cellular and molecular basis of this improvement is not known. It is possible that functional SERCA1 molecules accumulate over the course of development or that SERCA2, a

homolog of SERCA1 that is also expressed in muscle, can partially compensate for loss of SERCA1 (Ebert *et al.*, 2005). It is also possible that non-SERCA mechanisms develop to regulate cytoplasmic calcium levels. Examining calcium regulation and/or SERCA2 in *acc^{dta5}* mutants may enhance our understanding of muscle function.

Mutation of *atp2a1* has been shown to disrupt muscle function in a variety of species. In humans, both missense and presumptive null mutations in *ATP2A1* result in Brody disease, a condition in which individuals exhibit a normal life span but slowed relaxation of skeletal muscle (Periasamy and Kalyanasundaram, 2007). Deletion of *Atp2a1* in mice results in a more severe impairment of skeletal muscle function than in humans. Knockout mice die soon after birth due to diaphragm muscle hypercontractility (Pan *et al.*, 2003). The *ATP2A1* gene has also been identified as mutated in two muscle-contraction disorders in two breeds of cattle, Chianina and Belgian Blue. The Chianina disorder results in a more mild, Brody disease-like muscle relaxation defect (Sacchetto *et al.*, 2009). The Belgian Blue disorder results in death a few weeks after birth due to respiratory complications, similar to the *Atp2a1* knockout mice (Charlier *et al.*, 2008). In human, mice, and cattle, the disorders that disrupt SERCA1 function seem to be recessive and no phenotype has reported in any of the heterozygotes, thus the semi-dominant *acc^{dta5}* mutant could provide a particularly sensitive model for SERCA1 disorders.

METHODS

Zebrafish Maintenance and Breeding

Zebrafish were raised and maintained in our fish facility according to established protocols. Embryos were staged as described in Kimmel *et al.* (Kimmel *et al.*, 1995). The *acc^{dta5}* and *acc^{tg206}* alleles were maintained in a Tuebingen/TL genetic background.

Behavioral Analysis

To examine embryos at 24 hpf, touch stimuli was applied using a probe and the responses were examined using a dissecting microscope. The percentage of embryos that perform accordion-like behavior was determined by examining populations of wild-type, *acc^{dta5}* and *acc^{tg206}* without knowledge of genotype.

To examine swimming behavior at 48 hpf, touch stimuli was applied using a probe and the responses were recorded using a high-speed video camera recording at 1000 frames/second (TroubleShooter 1000 Fastec Imaging). Kinematic analysis was performed by measuring the head-to-tail angle during each frame using a custom designed computer program. Angles were calculated using three landmark points along the body: the head, the caudal end of the yolk, and the tip of the tail. The angles were graphed over time using Microsoft Excel.

To perform time-trial assays, single 48 hpf zebrafish larvae were placed at the center of two concentric circles. The distance between the inner and outer circle was 4mm. The larvae were gently touched on the head and the escape response was recorded using the high-speed video camera recording at 1000 frames/second. The amount of time was measured from when the larval head crossed over the boundary of the inner circle and touched the outer circle. Individual larvae were tested once, entire clutches were assayed, and each genetic cross was performed multiple times. The results were compiled into graphs using DeltaGraph. Statistical analysis was performed to compare the average swim times between all genetic crosses using the student's t-test.

Chromosomal Mapping and cDNA Analysis

Crosses between Tuebingen/TL strain fish carrying the *acc^{dta5}* allele and polymorphic WIK fish were used to generate a three-generation map cross panel using standard techniques. F2 *acc^{dta5}* homozygous mutant and wild-type sibling embryos were collected, sorted based on the motility phenotype, and stored in MeOH at -20°C . Bulk-segregant analysis was performed at the Zebrafish Mutant Mapping Facility at the University of Louisville. DNA was extracted from pools of 20 mutant and 20 wild-type embryos and analyzed using a variety of CA-repeat markers. To determine the *acc^{dta5}* linkage interval, several nearby markers on chromosome 3 were used with DNA from 96 individual F2 mutant embryos.

To clone *atp2a1* cDNA, RNA was extracted from 48hpf wild-type, *acc^{dta5}* heterozygous and *acc^{dta5}* homozygous embryos and used for RT-PCR. The Accuscript RT-PCR system (Stratagene) was used with oligo-dT and gene-specific primers to reverse transcribe and amplify *atp2a1*. The PCR products were gel purified, sequenced, and the nucleic acid sequences were analyzed using MacVector 9.0. RT-PCR was performed and analyzed multiple times with each genotype to decrease the possibility of amplification artifacts.

In Situ Hybridization

In situ hybridization was performed using established protocols (Thisse and Thisse, 2008). The *atp2a1* probe was generated by using RT-PCR to amplify a 572bp region at the 3' end of *atp2a1*. This fragment was subcloned, linearized, and used for *in vitro* transcription using the digoxigenin RNA labeling kit (Roche). A compound microscope attached to a digital camera (Zeiss) was used to acquire images, and the pictures were processed using Autostitch, Adobe Photoshop and Illustrator.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *dta5* exhibits a semi-dominant mode of inheritance

Selected, superimposed frames from a high-speed video camera illustrate escape behaviors of (a) wild-type, (b) heterozygous, and (c) homozygous mutant larvae at 48hpf. Gentle touch with a probe was used to stimulate escape behavior, and it has been removed from each frame for clarity. The duration of each response is indicated in milliseconds (ms). (d–f) Kinematic traces are shown for 48hpf larvae. For each condition, five representative traces are shown from the first 250ms of a response. Each response is from a different larva. The body angles are shown in degrees with 0 indicating a straight body, and positive and negative values indicating body bends in opposite directions. Note that mutants carrying one *dta5* allele display a reduced number of body bends and ability to swim. Swimming behavior is further reduced in mutants with two *dta5* alleles.

Figure 2. Wild-type and *dta5* mutant larvae increase swimming speeds over the course of development

The amount of time for each larva to swim 4mm was measured. The total number (*n*) of larvae tested, the average speed in mm/s (*a.s.*), and the average time (*a.t.*) for all of the larvae that can swim are indicated. Larvae that do not swim are not included in the average speed or time. The percentages of larvae from the total number of tested are plotted in time bins, with numbers indicating the upper limit of a time bin. 50ms bins are shown to the left of the dashed line and 250ms bins are shown to the right of the dashed line. Embryos that do not swim are plotted in the time bin furthest to the right. (a) All wild-type larvae swim the 4mm distance within 500ms at 48hpf. Wild-type embryos swim the 4mm distance increasingly faster at (b) 96hpf and (c) 144hpf. (d) Crosses between *dta5* heterozygotes yield a population of larvae in which some swim with wild-type times, defined as faster than 500ms, some heterozygotes swim slowly, and a group of mostly homozygotes do not swim at all. Regardless of genotype, most larvae have acquired the ability to swim at (e) 96hpf and (f) 144hpf.

Figure 3. The *dta5* mutation disrupts *atp2a1*/SERCA1, a calcium ATPase important for muscle relaxation

(a) A portion of chromosome 3 is shown with map positions, CA-repeat markers, and the location of the *Atp2a1* gene indicated. *dta5* maps to a large 0 recombinant interval (from 92 mutants) from marker z59380 to z68358. (b) Chromatogram sequence traces, with nucleotide sequence above and translated sequence below, from wild-type, *dta5* heterozygous, and *dta5* homozygous embryos. A single mutation was observed, which segregates along with the phenotype, and results in the substitution of Gly598 to Val. (c) Alignment of the Gly598 region of SERCA1 from different species demonstrates its precise conservation. Shading indicates amino acid identity and the arrow indicates Gly598. Gly598 is also conserved among the other two SERCA-encoding genes in humans and single orthologs in *Drosophila* and *C. Elegans*. (d) A schematic of the SERCA1 crystal structure illustrates the locations of SERCA1 functional domains, the *accordion* mutations previously identified in zebrafish (white X), and the *dta5* mutation (red X with arrow). The schematic is adapted from (Toyoshima and Inesi, 2004).

Figure 4. *acc^{dta5}* is a semi-dominant allele of *acc*

Time-trial assays of various crosses are shown. Crosses involving mutants consist of mating heterozygous adults since homozygotes do not survive to adulthood. All larvae were assayed at 48hpf. The total number of larvae and average swimming times are indicated. The calculation of swimming times and depiction of time bins are identical to Figure 2. **(a)** Wild-type (WT) larvae swim quickly, with nearly all (99%) swimming 4mm in less than 500ms. **(b)** Crosses between *acc^{dta5}* heterozygous adults yield larvae that swim with wild-type swim times, those that swim slowly, and those that do not swim at all. The WT and *acc^{dta5}* crosses shown here are different populations than those in Figure 2 and illustrate the consistent results obtained using this assay. The differences in the average times between this and Figure 2 are not statistically significant. **(c)** Crosses between *acc^{tq206}* heterozygotes produce larvae that swim quickly, few that swim slowly, and larvae that do not swim. **(d)** Crosses between wild-type and *acc^{dta5}* heterozygotes yield populations that swim with wild-type times and those that swim slowly, illustrating the semi-dominant effect. **(e)** Crosses between wild-type and *acc^{tq206}* heterozygotes reveal larvae that swim like wild-type and those that swim slightly slower, indicating a subtle semi-dominant effect. **(f)** Crosses between *acc^{tq206}* and *acc^{dta5}* show a failure to complement, which demonstrates that they are allelic to each other.