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Novel role for carbamoyl phosphate synthetase 2 in cranial sensory circuit formation

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ABSTRACT

In zebrafish, cranial sensory circuits form by 4 days post-fertilization. We used a forward genetic screen to identify genes involved in the formation of these circuits. In one mutant allele, *sl23*, axons arising from the epibranchial sensory ganglia do not form their stereotypical terminal fields in the hindbrain. These embryos also had small eyes and deformed jaws, suggesting a pleiotropic effect. Using positional cloning, a 20-nucleotide deletion in the carbamoyl-phosphate-synthetase2-aspartate-transcarbamylase-dihydroorotase (*cad*) gene was found. Injection of a CAD morpholino phenocopied the mutant and mutants were rescued by injection of *cad* RNA. Cad activity is required for pyrimidine biosynthesis, and thus is a prerequisite for nucleic acid production and UDP-dependent protein glycosylation. Perturbation of nucleic acid biosynthesis can result in cell death. *sl23* mutants did not exhibit elevated cell death, or gross morphological changes, in their hindbrains. To determine if defective protein glycosylation was involved in the aberrant targeting of sensory axons, we treated wild type embryos with tunicamycin, which blocks N-linked protein glycosylation. Interference with glycosylation via tunicamycin treatment mimicked the *sl23* phenotype. Loss of *cad* reveals a critical role for protein glycosylation in cranial sensory circuit formation.

1. Introduction

Survival of an organism is dependent upon the correct wiring of its neural circuits. In vertebrates, the cranial sensory circuits are vital, as they provide essential information from a wide variety of stimuli and organs, e.g., pain, touch and temperature from the head/throat, taste, blood pH and arterial pressure, and organ distension originating from thoracic and abdominal viscera. Many of the cranial nerves that carry afferent sensory information also contain efferent motor axons, and all cranial nerves contain glial elements. We have previously shown in zebrafish that sensory axon pathfinding from the epibranchial ganglia to the hindbrain involves interactions with both branchiomotor axons and peripheral glia and occurs during the first four days post-fertilization (dpf) (Cox et al., 2011). The mechanisms underlying these cell–cell interactions are poorly understood.

Very little is known about the gene networks responsible for establishing the connectivities of these circuits. However, the zebrafish offers the opportunity to identify genes involved in developmental processes, in an unbiased manner, using forward genetic screens (Driever et al., 1996). We have taken advantage of this attribute by engineering a transgenic zebrafish line *Tg(p2xr3.2:gfp)* that expresses eGFP (green fluorescent protein) in nearly all peripheral sensory neurons, beginning in embryogenesis and continuing into adulthood (Kucenas et al., 2006). The eGFP fills the cell body together with its processes, allowing for visualization of the limbs of sensory circuits as they form and are maintained in the fish. Using this line, we carried out an ethylnitrosourea-based forward screen to identify genes involved in cranial sensory circuit formation. In this paper, we describe one mutant allele, *sl23*, in which the terminal fields of the central projections of the facial (gVII), glossopharyngeal (gIX) and vagal (gX) ganglia do not form properly. Using positional cloning techniques, we have identified the *sl23* mutation as a deletion within the gene encoding the carbamoyl-phosphate-synthetase2-aspartate trancarbamylase-dihydroorotase (Cad) enzyme, which results in a null protein. This enzyme is responsible for the rate limiting step in the pyrimidine biosynthesis pathway and is essential for the production of the UDP-sugars required for protein glycosylation (Jones, 1980). The results presented here indicate that defective protein glycosylation plays a major role in the

Abbreviations: cad, carbamoyl-phosphate synthetase 2-aspartate transcarbamylase-dihydroorotase; *sl23*, *Tg(p2xr3.2:gfp)*^{sl23}; *plx*^{a52}, perplexed mutant; UPR, unfolded protein response; bmn, branchiomotor neurons.

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sensory axon malformations seen in these mutants, although defects in nucleic acid synthesis leading to perturbations in cell cycle and cell death may also contribute to the observed phenotype.

2. Materials and methods

2.1. Maintenance of fish

Fish were kept on a 14-h day, 10-h night schedule at a constant 28.5 °C with feeding done twice daily. All animal husbandry was carried out as described by Westerfield (2000). Embryos were staged according to hours post-fertilization (hpf) and morphological criteria (Kimmel et al., 1995). Embryos used for microscopy were treated with 0.003% phenylthiourea to reduce pigmentation. The Tg(*p2xr3.2:eGFP*) line has been previously described (Kucenas et al., 2006, 2009) and Tg(*isl1:eGFP*) (Higashijima et al., 2000) fish were a gift from H. Okamoto.

2.2. Imaging of embryos/larvae

Epifluorescent microscopy was carried out on embryos/larvae that were anesthetized with 0.01% Tricaine (ethyl 3-aminobenzoate methanesulfonate salt) in fish water and transferred to a 96-well plate. Images were obtained using a Nikon TE200 inverted microscope equipped with a CoolSNAP HQ digital camera. MetaMorph software (Universal Imaging Corp) was used to acquire and process images. Cropping and rotating of images was carried out using Adobe Photoshop. For confocal microscopy, embryos/larvae were embedded in 1% low-melting point agarose containing 0.01% Tricaine. Imaging was performed with an Olympus FV1000 MPE using a 20×/0.95 water immersion objective. Images collected in the *z*-dimension were collapsed into one maximal intensity projection or were rendered into 3-dimensions using either Olympus Fluoview or NIH ImageJ software. Final brightness and/or contrast values of images were adjusted in Adobe Photoshop CS3.

2.3. Alcian blue staining

5 dpf larvae were fixed overnight at 4 °C in 4% paraformaldehyde/PBS. They were then dehydrated with sequential incubations in 50% ethanol and then 100% ethanol. Embryos were incubated overnight in 0.1% Alcian blue solution in 70% ethanol/30% acetic acid, neutralized in saturated sodium borate solution for 2 h, digested in trypsin (2 mg/ml in 30% saturated borate) at 37 °C for 1 h and then bleached in 5% hydrogen peroxide/1% KOH for 2–3. They were then washed with PBS and fixed in 4% paraformaldehyde/PBS at 4 °C overnight. For imaging, embryos were embedded in 1% agarose and viewed using an Olympus BX60 upright microscope equipped with an Olympus DP71 digital camera. Olympus software was used to acquire and process images. Cropping and rotating of images was done in Adobe Photoshop.

2.4. Embryo injections

RNA or morpholino oligonucleotide (MO) was injected into single-cell embryos using a Picospritzer III (General Valve Corporation, Fairfield, NJ) attached to a broken glass capillary. Full length Cad cDNA was subcloned into pCR-Blunt (Invitrogen) and linearized with Spe I. Capped RNA was transcribed in vitro using the T7 Amplicap kit (Epicenter). The RNA was dissolved in diethylpyrocarbonate-treated distilled H_2O and mixed 1:1 with injection buffer (0.1 M KCl, 20 mM HEPES (pH 7.4) and 0.01% Phenol Red). A 1 mM solution of Cad morpholino (TAAAGATGCCATTTTCAGCGACATG) (Willer et al., 2005), which overlaps the start codon, was heated at 65 °C for 10 min, to ensure it was in solution. The solution was then diluted to 500 μM with distilled H_2O and mixed 1:1 with injection buffer, to give a 250 μM solution (~2.5 ng/nl).

2.5. Positional cloning

Standard mapping methods using bulk segregant and meiotic recombination analysis of microsatellite markers (Green et al., 2009) were used to map the sl23 mutation to chromosome 20. Total RNA was then extracted from mutant and wild type 4 dpf larvae and cDNA synthesized with Superscript III reverse transcriptase (Invitrogen). Sequences corresponding to candidate genes were then obtained by PCR and sequenced using gene specific primers.

2.6. Immunohistochemistry

Embryos/larvae were fixed in 4% paraformaldehyde/PBS at 4 °C overnight and then washed 3× for 5 min in PBS–1% TritonX-100 (PBT). The larvae were then treated with 0.25% trypsin in PBS on ice for 10–15 min and then washed 3× for 10 min in PBT. After incubation in a blocking solution (2% normal goat serum/1% DMSO/2 mg/ml BSA in PBT) for 1 h at room temperature, the embryos were incubated with mouse anti-acetylated tubulin (1:1000) (Sigma), mouse anti-zrf-1 (1:500) (ZIRC) or rabbit anti-phospho-histone 3 (1:750) (Santa Cruz) antibody overnight at 4 °C. Embryos were then washed in PBT for 3 h with at least 4 changes and then incubated with rabbit anti-mouse or goat anti-rabbit Alexa 568-conjugated secondary antibodies (1:1000) (Invitrogen) overnight at 4 °C. Embryos were then washed extensively and maintained in PBS at 4 °C for up to a week. Images were obtained by confocal microscopy as described above.

2.7. Acridine orange assay

Live dechorionated embryos are incubated in a 2 μg/ml solution of acridine orange (Sigma) in PBS for 20 min at room temperature. Embryos are then washed 4× 1 min in fish water and then visualized using epifluorescence.

2.8. Tunicamycin treatment

Tunicamycin (Sigma) was dissolved in fish water at a stock concentration of 1 mg/ml in DMSO. Embryos at the 16 hpf stage were placed into a 96 well plate and the fish water replaced with new water containing tunicamycin at a final concentration of 1 μg/ml. Embryos were kept in drug for 24 hpf, at which time they were washed and imaged by epifluorescence microscopy.

3. Results and discussion

We have carried out a forward genetic screen to identify genes involved in branchiomeric sensory axon pathfinding, and have identified a number of mutant lines that displayed perturbations in sensory circuits. One of these alleles, *sl23*, exhibited defective circuit formation of the epibranchial afferent nerves (Fig. 1). When live larvae were examined using confocal microscopy, all epibranchial ganglia were found in their stereotypical positions around the ventral margins of the otic vesicle, although the facial (gVII), glossopharyngeal (gIX) and vagal (gX) ganglia appeared reduced in size when compared to wild-type *Tg(p2xr3.2:gfp)* larvae (Fig. 1A and B). The sensory axons from the VIIth and IXth ganglia projected to the hindbrain following their normal trajectories; however, once in the CNS, perturbations in afferent axon pathfinding within the hindbrain were observed, with the epibranchial axons unable to form the stereotypical hindbrain plexus seen in wild type embryos ($n = 1062$ mutants) (Fig. 1B). In some cases, the IXth axons are very faint and difficult to observe, as there appears to be less axons in the mutant compared to wild type and their trajectory take them close to the axons of the Xth. The sensory axons of the Xth do not defasciculate into their typical branching pattern as they course alongside the external surface of the hindbrain, but instead remain as one or a few bundles as they enter the CNS. Again, their terminal fields in the hindbrain are malformed.

In addition to the epibranchial nerve defects, *sl23* homozygotes possessed other developmental dysmorphisms: they were smaller than their wild-type clutch mates, had smaller eyes (Fig. 1C) and died between 8 and 10 days post-fertilization. In addition, their pharyngeal cartilages were malformed, with inverted and reduced ceratohyals, deformed Meckels cartilages and a loss of ceratobranchials (Fig. 1D). Pigment formation was not affected. Together, these findings suggested that the mutated gene in *sl23* is not specific to axon guidance, but instead functions in a pleiotropic manner across many cell types.

3.1. sl23 is a truncated CAD

Genetic analysis demonstrated that the *sl23* mutation was recessive and possessed a Mendelian inheritance pattern: on average, mutants comprised 29 ± 2% of larvae in a clutch ($n = 908$, from 6 clutches). Using positional cloning, we localized the *sl23* lesion to a region of chromosome 20 (20:38,670,000–39,330,000) that contains all or parts of the *mpv17*, *trim54*, *cad*, *ift172*, *msra*, *rcan* and *reps1* genes (Fig. 2A). Sequencing of candidate genes in this critical interval revealed a 20 bp deletion in exon 5 of the carbamoyl-phosphate synthetase 2-aspartate transcarbamylase-dihydroorotase (*cad*) gene (Fig. 2B). Although insertions and deletions are not common in zebrafish ENU screens, they have been reported in other systems (Watson et al., 1998). This gene encodes a multifunctional 2230 amino acid protein (Fig. 2C) whose activities comprise the first three steps in *de novo* pyrimidine biosynthesis (Sigoillot et al., 2002) and which is responsible for the rate-limiting step in this pathway. The observed deletion excises base pairs