Analysis of the zebrafish *perplexed* mutation reveals tissue specific roles for de novo pyrimidine synthesis during development

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ABSTRACT

The zebrafish perplexed mutation disrupts cell proliferation and differentiation during retinal development. In addition, growth and morphogenesis of the tectum, jaw, and pectoral fins are also affected. Positional cloning was used to identify a mutation in the carbamoyl-phosphate synthetase2-aspartate transcarbamylase-dihydroorotase (cad) gene as possibly causative of the perplexed mutation and this was confirmed by gene knockdown and pyrimidine rescue experiments. CAD is required for de novo biosynthesis of pyrimidines that are required for DNA, RNA, and UDP-dependent protein glycosylation. Developmental studies of several vertebrate species showed high levels of cad expression in tissues where mutant phenotypes were observed. Confocal time-lapse analysis of perplexed retinal cells in vivo showed a near doubling of the cell cycle period length. We also compared the perplexed mutation with mutants that affect either DNA synthesis or UDP-dependent protein glycosylation. Cumulatively our results suggest an essential role for CAD in facilitating proliferation and differentiation events in a tissue specific manner during vertebrate development. Both de novo DNA synthesis and UDP-dependent protein glycosylation are important for the perplexed phenotypes.
INTRODUCTION

We have used a forward genetic approach in zebrafish to identify and study genes essential for retinal development. The neural retina of zebrafish, as in other vertebrates, arises from neuroepithelial cells that line the optic cup. All of these elongated cells rapidly divide through multiple rounds of mitosis before the first group of progenitor cells leave the cell cycle. At the time of cell cycle exit, retinal progenitor cells undergo cell type fate decisions, initiate post-mitotic cell migration, and begin to differentiate into one of the seven major cell types found in vertebrate retinas (reviewed in OHNUMA et al. 2001; LEVINE and GREEN 2004). In fish and other ectothermic vertebrates, the retina continues to grow throughout larval development from a population of slowly proliferating stem cells found at the outer margin of the neural retina. Fundamental questions in retinal development, and developmental biology in general, center on the coordination of cell proliferation, cell cycle exit, and differentiation of progenitor cells.

The perplexed mutation was identified in a genetic screen for ethynitrosourea (ENU) - induced mutants that showed disrupted retinal lamination in the absence of gross embryological defects (LINK et al. 2001). Embryos homozygous for the perplexed mutation have small eyes and a reduced tectum and die between 8-12 days post fertilization (dfp). Our previous analyses indicated that the perplexed gene is essential for the transition from a proliferative to a post-mitotic state within the neural retina. Elevated cell death was observed at the time when retinal progenitor cells normally begin to withdraw from the cell cycle. In addition, retinal differentiation and morphogenesis was dramatically diminished in surviving cells within perplexed retinas. Genetic mosaic analysis indicated that the mutation is non-cell-autonomous for both survival and differentiation. As part of the current study, we have used positional cloning and other experiments to identify and confirm that a mutation in the gene encoding the trifunctional enzyme carbamoyl-phosphate synthetase2-aspartate transcarbamylase-dihydroorotase (CAD) is responsible for the perplexed phenotypes.

CAD is the rate limiting enzyme for de novo biosynthesis of pyrimidine-based nucleotides and catalyzes the first three steps in de novo pyrimidine biosynthesis (reviewed in JONES
1980; Figure 1). Utilizing ATP, CAD converts glutamine and bicarbonate to dihydroorotate for the production of orotate. Orotate is then rapidly converted to uridine-5’-diphosphate (UDP), which is a precursor for production of cytosine and thymine based nucleotides, and ultimately RNA and DNA synthesis. UDP is also a precursor of UDP-sugar intermediates, which are required for post-translational modification of many proteins. The function of CAD, therefore, is important for both de novo RNA and DNA synthesis and for specific types of protein glycosylation. In most cells, UDP and other nucleotides can also be provided by the salvage pathways. Several cultured cell lines lack functional CAD, but can be maintained in a pyrimidine-supplemented medium (DAVIDSON and PATTERSON 1979; PATTERSON et al. 1992; QUI and DAVIDSON 1998). The utilization of de novo versus salvage pathways for pyrimidine biosynthesis in vivo has been proposed to be dependent on tissue and cell type, as well as the mitotic state of a cell (ANDERSON and PARKINSON, 1997).

In Drosophila a mutation in CAD, rudimentary, was among the first described by Thomas Hunt Morgan and was used for discovering meiotic recombination (MORGAN, 1913; STURTEVANT 1913). Flies with the rudimentary mutation show malformed and reduced wings as well as female sterility (MORGAN 1911; MORGAN 1918). The mutation is lethal only when larvae are grown on pyrimidine-free medium. While these results show that CAD has tissue specific functions during invertebrate development, the role of CAD in vertebrate development has not been investigated.

In the current study, we have used positional cloning, gene knock-down, and pyrimidine rescue experiments to identify and confirm a mutation in the zebrafish cad gene as causative for the perplexed mutation. To address tissue specificity we have investigated cad gene expression during development in several vertebrate species. Finally, we have further analyzed the retinal and non-retinal phenotypes of perplexed by comparing cad mutants to embryos with mutations in other genes required for either nucleotide synthesis or UDP-glycosylation. Cumulatively our results suggest an essential and complex role for CAD in facilitating proliferation and differentiation events in a tissue specific manner during vertebrate development. Our observations are consistent with the hypothesis that basic
metabolic pathways are key targets of regulatory signals essential for coordinating cellular proliferation and differentiation during development.

**MATERIALS AND METHODS**

**Mutant alleles:** The following mutant alleles were used in this study. The gene affected is listed in brackets and the original citation in parentheses. *perplexed*<sup>a52</sup> [cad] (LINK et al. 2001); hi<sup>688</sup> [ribonucleotide reductase R2], and hi<sup>954</sup> *[UDP-glucoronic acid decarboxylase]* (GOLLING et al. 2002); hi<sup>2694</sup> [cad], hi<sup>3378</sup> *[UDP-glucoronic acid/UDP-N-acetylglactosamine dual transporter]*, and hi<sup>3510</sup> [thymidylate synthase] (AMSTERDAM et al. 2004).

**Positional Cloning:**

*Bulked segregant linkage analysis:* The recessive *perplexed*<sup>a52</sup> mutation was outcrossed with wild type AB fish and the mutant line propagated by repeated AB outcrossings (LINK et al. 2001). Since meiotic recombination rates are lower in male zebrafish compared to females, the use of male meiosis is favorable for bulked segregant analysis to identify on which chromosome the mutation is located (SINGER et al. 2002). A mapping panel to maximize the information content of the male parent was generated by outcrossing an AB +/- *perplexed* fish with a TL +/- fish and then backcrossing a resulting TL+/+ *perplexed* heterozygous male with an AB +/- *perplexed* female. Genomic DNA was isolated from homozygous *perplexed* mutant embryos and wild-type siblings and two pools of 20 mutant and 20 wild-type embryos were used for bulk segregant analysis. Simple sequence-length polymorphism markers (KNAPIK et al. 1998; SHIMODA et al. 1999; http://zebrafish.mgh.harvard.edu/zebrafish/index.htm) spaced ≈20 centimorgans (cM) apart across the length of the genome were amplified by polymerase chain reaction (PCR) and the products analyzed on 3% agarose gels. Once linkage was detected, 96 individual mutants were genotyped to confirm linkage and to refine the critical interval.

*High resolution linkage mapping:* Given that increased rates of recombination are more advantageous for high resolution linkage mapping, new mapping panels informative for recombination in the female parents were generated. AB +/- *perplexed* fish were outcrossed
to WIK +/+ fish and resulting WIK+/perplexed were mated to AB +/perplexed to generate homozygous perplexed mutants for fine mapping. Fluorescent primers (Prologo, Boulder, CO) were designed for markers Z8150 and Z9794 and 530 mutant embryos were genotyped on a CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton, CA) to identify recombinant embryos. BLAST searches were used to identify finished BAC clones from the NCBI database (http://www.ncbi.nlm.nih.gov/genome/seq/DrBlast.html) and scaffolds from the zebrafish assembly version 4 (http://www.ensembl.org/Danio_rerio/) that were located in the critical region. SeqMan II software (DNASTAR, Madison, WI) was used to assemble the BAC clones and scaffolds into a single contig that spanned the entire length of the critical interval containing the perplexed mutation. Single nucleotide polymorphisms (SNPs) producing restriction fragment length polymorphisms (RFLP) were identified by PCR amplifying, cloning, and sequencing fragments across the region from the female parent. These new informative markers were used to refine the critical interval.

Candidate gene search: Candidate genes within the perplexed critical region were identified using GENSCAN (http://genes.mit.edu/GENSCAN.html). Total RNA was isolated from pools of 100 perplexed and wild type embryos using a VERSAGENE RNA Purification Kit (Gentra Systems, Minneapolis, MN) and cDNA synthesized using oligo dT and Superscript II as described by the manufacturer (Invitrogen, Carlsbad, CA). The full length ORF representing the cad gene was amplified using primers (forward, 5’-AGGGACAGCATGCTATTTGG-3’; and reverse, 5’-TGGTCCCAATTGGATAGGAA-3’) and the Accuprime Taq polymerase as described by the manufacturer (Invitrogen, Carlsbad, CA). Amplified products were cloned, sequenced, and analyzed for potential mutations. The identified T to G transversion introduces an Ava II restriction enzyme site, which was subsequently used to confirm the mutation was present in genomic DNA from perplexed embryos.

Orotic acid and Uridine Rescue: The sodium salts of orotic acid (#O3000) and uridine (#U0750) were obtained from Sigma (St. Louis, MO). Stock solutions made in embryo medium (WESTERFIELD 1995) and 50 nanoliters of various concentrations (1µM-10 mM) were injected into the yolk sack of developing wild type and perplexed embryos at 20 hours
post fertilization (hpf). Partial rescue of eye, jaw and fin development was observed at a minimal concentration of 1 µM for orotic acid and 100 µM for uridine. Concentrations higher than 1000 µM resulted in early developmental lethality. To evaluate retinal histology, embryos were fixed in 4% paraformaldehyde at 72 hpf and processed for cryosectioning. Sections were mounted on glass slides and nuclei were stained with Hoechst 33258 (0.5 µg/ml in PBS).

**Morpholino Antisense Knockdown:** We designed 3 independent morpholino antisense oligonucleotides (GeneTools, Philomath, OR) to the zebrafish cad gene. CADMO1 (TTAATTTTCACCTACCAACTTCACC) overlapped exon 1 donor sequence and used at 40 µM. CADMO2 (AAACAAAAATAACCTTGCTGAGTC) overlapped exon 2 donor sequence and was used at 30 µM. CADMO3 (TAAAGATGCCATTTTCAGGCACATG) overlapped the ATG start site and was used at 200 µM. These oligonucleotides were diluted in sterile water with 0.02% phenol red for visualization purposes and 15 nanoliters was injected into one- or two-cell stage wild type embryos. As controls, Standard Control morpholino (GeneTools) was injected at equivalent concentrations. To confirm efficacy of splicing inhibition, a subset of embryos injected with MO1 and MO2 were taken for reverse transcription – polymerase chain reaction (RT-PCR) analysis (DRAPER et al. 2001). For MO1 and MO2, 30 control and 30 CADMO1- or CADMO2 -injected embryos were collected at 32 or 48 hpf for RT-PCR analysis. A forward primer specific to exon1 (CADEX1F1 GTCTGTTCCGTGCGGTATCT) and a reverse primer specific to exon3 (CADEX3R1 GCTCTCTGAGCCACTCATCC) were used to amplify the cDNA pools. Analysis indicated that both MOs induce alternative splicing events that disrupt gene function.

**Histology:** Semi-thin retinal sections were obtained after fixing embryos overnight at 4°C in 2.5% gluteraldehyde/1% paraformaldehyde/phosphate-buffered sucrose, pH 7.4. Embryos were dehydrated and infiltrated with Epon/Araldite. Transverse sections, 1-2 µm, were heat mounted and stained with 1% methylene blue in 1% borax.
**Alcian Blue Cartilage staining:** PTU-treated embryos (approximately 15) were placed in 1.5 ml plastic centrifuge tubes and fixed in 1 ml of 3.7% neutral buffered formaldehyde at room temperature for 2 hrs. Embryos were then rinsed in PBS and transferred to Alcian Blue Stain (0.1% Alcian Blue in 80% ethanol / 20% glacial acetic acid) for overnight incubation at room temperature. The next day, embryos were rinsed in 100% ethanol and gradually rehydrated in PBS. Further washes were carried out in 1% KOH in PBS. Embryos were mounted in 1% low melting agarose for photography.

**Riboprobes and In Situ Hybridization:** Localization of mRNA in zebrafish was performed on whole embryos (JOWETT and LETTICE 1994). For chicken and mouse, in situ hybridization was performed on paraffin sectioned embryos as described in LEE et al., 2005. Digoxegenin-labeled cRNA probes corresponding to the following regions of cDNA were used: zebrafish (6,365-7,000 bp, Accession AY_880246), chicken (43-3,043 bp, Accession XM_426217), mouse (6,139-6,930 bp, Accession NM_023525).

**Transgenesis and Time-lapse microscopy:** Wild type or perplexed mutant embryos were injected at the one- to four-cell stage with ~15 nl of 50 ng/µl circular plasmid DNA encoding histoneH2B::Green Fluorescent Protein (GFP) fusion protein (KOSTER and FRASER 2001). Embryos were grown in PTU to block pigment synthesis. At 32-34 hpf, embryos were anesthetized with 0.05% Tricane and embedded in 1.0% low melt agarose. Fish were oriented on their side within a 35 mm² culture dish (#P35G-1.5-10-C, MatTek Corp, Ashland, MA). Cells labeled with the transgene were then imaged with a Nikon C1 confocal microscope for 15-20 hours. Z-sections (40 µm total depth) were collected every 12 minutes. This time was determined empirically to be sufficient to capture M-phase for each cell. Temperature was maintained throughout all experiments at 28.5°C using a stage incubator.

**Cell cycle analysis:** Image planes from confocal time-lapse microscopy were converted from IDS to ND format using Metamorph Imaging software (Universal Imaging Corp, Philadelphia, PA). This data was then arrayed by time and z plane using the Multidimensional analysis tool suite. Individual cells were followed from M-phase to M-phase. M-phase was easily viewed by the condensed and elongated nature of the chromatin.
The time required for this was recorded as the total cell cycle period. If a cell underwent apoptosis, as evident by DNA fragmentation, the latency from the last M-phase was recorded. In all, we quantified the cell cycle period or latency to cell death for n=26 perplexed retinoblasts (4 time-lapse experiments) and n=27 wild type retinoblasts (3 time-lapse experiments).

RESULTS

Zebrafish embryos with the perplexed mutation show reduced eye size and lack retinal cell morphogenesis (LINK et al. 2001). Increased apoptosis, as compared to wild type siblings, is apparent by histology at 40 hours post fertilization (hpf). These phenotypes suggested that the perplexed gene was critical for normal proliferation and differentiation in the retina. Linkage mapping with microsatellite markers localized perplexed to chromosome 20, between z8150 and z9794. Recombinant mutant embryos were then used in conjunction with additional microsatellite markers and newly developed SNPs. Markers z4394 and z536 showed no recombinants out of 530 embryos genotyped. BACs and scaffolds (assembly version 4) were identified that spanned the entire critical region between z13672 and z8164 (Figure 2A). Genescan analysis of this region revealed two potential candidate genes, a selective LIM-binding factor homolog and cad. Cloning and sequencing of the cad open reading frame from perplexed and wild type embryos revealed a mutation at nucleotide position 3848. This T to G transition produces a non-conservative methionine to arginine substitution at position 1283 of the protein. Comparison of sequence surrounding M1283 in the carbmoyl synthetase 2 domain of cad, revealed that this methionine is conserved from humans to C. elegans (Figure 2B,C).

To confirm that the M1283R mutation in cad was responsible for the perplexed phenotype, we designed antisense oligonucleotides (‘morpholinos’) to either disrupt pre-mRNA splicing or to block translation (NASEVICIUS et al. 2000; DRAPPER et al. 2001). Wild type embryos injected with any of these morpholinos phenocopied the perplexed mutation (Figure 3). Whole embryo morphology and retinal histology show the similarities between perplexed and CAD morphant embryos. To further test whether the mutation in cad is responsible for
the *perplexed* phenotype, we attempted to rescue the phenotype by providing pyrimidine nucleotide precursors that are in the de novo biosynthetic pathway, but downstream of CAD. Injection of either orotic acid or uridine into 24 hpf mutant embryos was able to rescue the retinal defects of the *perplexed* mutation as judged by eye size and plexiform layer formation (Figure 4). Partial rescue of jaw and fin morphogenesis was also noted. Neither of the compounds had an affect on the development of wild type embryos (data not shown).

Finally, a recently reported insertional mutagenesis screen in zebrafish described a retroviral insertion into the *cad* locus (AMSTERDAM et al. 2004). We obtained this line, hi2694, for phenotype analysis and complementation tests. Embryos homozygous for the hi2694 allele showed a phenotype indistinguishable from the *perplexed* embryos and pairwise crosses between *perplexed* and hi2694 heterozygotes were non-complementing for the mutant phenotype. Cumulatively, these data demonstrate that a loss-of-function mutation in *cad* is responsible for the *perplexed* phenotype. The ability of supplied orotic acid or uridine to rescue *perplexed* mutant phenotypes also explains the non-cell-autonomous nature of the *perplexed* mutation. When mutant cells are placed within a wild type environment, the compounds downstream of CAD provide substrates for UDP synthesis within the *perplexed* cells. Orotic acid and uridine can be taken up by adjacent cells through free diffusion, specific transporters, or via gap junctions depending on cell type and local concentration (ANDERSON and PARKINSON, 1997).

In addition to showing reduced eye and tectal size, *perplexed* embryos also show dismorphic and reduced fins as well as malformed jaw structures. However, other aspects of embryogenesis appear normal. To explore the tissue specificity of the *perplexed* phenotype, we investigated the developmental expression of *cad* mRNA. We found that *cad* transcripts were provided to the egg maternally (Figure 5). When zygotic transcription begins in zebrafish (~512 cell stage), *cad* expression was not spatially restricted. However, by the 18 somite stage, *cad* mRNA expression is down-regulated in the posterior region of the embryo and up-regulated in the CNS with particularly high expression levels in the retina and tectum. This trend continues until approximately 36 hpf. At this time, expression within the eyes and tectum becomes restricted to cells within the proliferative germinal zones. By 48 hpf,
transcripts for cad become prominent within the branchial arch regions, which will give rise to jaw structures, and within the developing fin buds. Moderate levels of cad expression is found within the liver, pancrease and intestine at 3-4 dpf, a time when cad expression in the tectum, retina, and branchial arches has been down-regulated. Overall, high levels of cad expression correspond to cells undergoing rapid proliferation or initiating differentiation, consistent with the dismorphic phenotypes of perplexed mutants.

To address whether the cad expression pattern in zebrafish is conserved in other vertebrates, we examined localization of cad transcripts by in situ hybridization in chicken and mouse embryo sections. Similar to zebrafish, the retina and tectum of chicken and mouse embryos showed high levels of expression during proliferative stages, with subsequent decreases following cell cycle exit. At the early developmental times, before cell cycle exit has commenced in either zebrafish, chick, or mouse, cad expression was uniform across the neural retina (Figure 6A-C). In the 84 hpf zebrafish retina, cad expression was maintained in the marginal zone where retinoblasts continue to proliferate (Figure 6D). In the stage 38 chick retina, when lamination has been established, cad expression has been down-regulated uniformly (Figure 6E). However, in the P3 mouse retina, increased cad expression in still observed in the inner nuclear layer where late proliferating bipolar and Müller cells reside (Figure 6F).

Several studies in cell culture have suggested an important role for pyrimidine nucleotides in modulating the rate of cell proliferation (HUISMAN et al. 1979; COLQUHOUN and NEWSHOLME, 1997; SIGOILLOT et al. 2004). In addition, the activity of CAD is modulated throughout the cell cycle with the highest activities found in S-phase (MORFORD et al. 1994; SIGOILLOT et al. 2002). Our previous studies with the perplexed mutation showed that the proportion of retinal cells in S-phase was dramatically increased at a time when retinal cells in wild type embryos were post-mitotic. This result could be due to either an increased cell cycle period or a delay in cell cycle exit of retinal progenitors. To address this, we used a time-lapse imaging technique to directly measure cell cycle parameters in living embryos. To label cells for imaging, wild type or mutant embryos were injected at the 1-4 cell stage with a plasmid encoding a fusion protein of HistoneH2B and GFP (KÖSTER
and FRASER 2001). This manipulation allows for mosaic expression of the transgene, such that individual cells can be followed from mitosis to mitosis via confocal timelapse microscopy. Figure 7 shows a time series of either a wild type (Figure 7A) or a perplexed (Figure 7B) retinal neuroepithelial cell completing one full cell cycle. In vertebrate neuroepithelial cells, the nucleus moves from the apical to basal surface – a behavior known as interkinetic nuclear migration. Our time-lapse analyses demonstrate that the average cell cycle period of perplexed retinal cells was twice as long as that of wild type retinal cells (Table 1). Interkinetic nuclear migration slowed proportionately to the cell cycle delay in perplexed, but no differences in M-phase kinetics were noted. We also calculated the proportion of dying cells and the latency from M-phase to nucleus fragmentation. No cell death was observed in GFP-labeled wild type cells, consistent with the low levels of normal apoptosis previously described for the zebrafish retina (BIEHLMAIER et al. 2001). In contrast, 25% of the retinal cells labeled in perplexed retina underwent apoptosis and this typically occurred within 2 hrs from the last cell division. These observations provide in vivo and genetic evidence that CAD function is essential for normal cell cycle progression.

CAD activity is required for de novo UDP biosynthesis, which is required for both DNA synthesis and post-translational modification. To address whether disruption of one or the other of these pathways was more or less responsible for the perplexed phenotype we analyzed several additional zebrafish mutants, all of which had been produced by retroviral insertion (GOLLING et al. 2002; AMSTERDAM et al., 2004). The mutant hi688 has an insertion in ribonucleotide reductase R2 (GOLLING et al. 2002). The other mutant, hi3510, has an insertion in the thymidylate synthase gene (AMSTERDAM et al., 2004).

Ribonucleotide reductases are composed of subunits R1 and R2. This enzyme catalyzes the synthesis of deoxyribonucleotides from their corresponding ribonucleotides. Therefore, ribonucleotide reductase provides the only source of precursor nucleotides – from either de novo or salvage pathways -- for DNA synthesis (JORDAN and REICHARD, 1998). Thymidylate synthase catalyzes the methylation of dUMP to dTMP and is essential for de novo synthesis of thymidine, which is also essential for DNA synthesis.
To examine if the lack of nucleotides for DNA synthesis was likely to be the main cause of the *perplexed* phenotypes, particularly given the extent to which the cell cycle in retinal cells was affected, we examined development in these mutants. The *ribonucleotide reductase R2* mutant showed very severe deficits and early embryonic lethality and could not be analyzed further. Thymidylate synthase mutants, which affect de novo DNA synthesis, but not UDP-dependent glycosylation, show reduced eye size. Interestingly, retinal lamination and differentiation appear normal as compared to *perplexed* retinas (Figure 8). To assay jaw and fin formation and differentiation, alcian blue cartilage staining and histological inspections were conducted (Figure 9). Thymidylate synthase mutants showed normal jaw structures and fin morphogenesis (Figure 9C). In contrast, *perplexed* embryos showed severe dismorphogenesis of the jaws and fins (Figure 9D). The overall staining intensity of alcian blue was reduced in *perplexed* and notably absent were Meckel’s, cleithrum, and hyosymplectic cartilage.

To examine the role of UDP-dependent glycosylation we analyzed the hi3378 mutant, which lacks the UDP-glucuronic acid/UDP-N-acetylglactosamine dual transporter. This transporter is part of a large family of nucleotide sugar transporters where each member has specificity for a particular nucleotide-sugar compound (BULTER and ELLING 1999). In humans there are five UDP-sugar transporters (ISHIDA and KAWAKITA 2004). Mutations in these transporters prevent the movement of the nucleotide-sugar intermediates from the cytoplasm to the Golgi apparatus and therefore block a sub-set of UDP-dependant glycosylation events. We also investigated hi954 which has a mutation in the *UDP-glucuronic acid decarboxylase* gene. This enzyme is required for the conversion of UDP-glucuronic acid to UDP-xylose, which is used for synthesis of numerous glycoconjugates abundant in the extracellular matrix and on cell surfaces. Embryos homozygous for a retroviral insertion in the UDP-glucuronic acid/UDP-N-acetylglactosamine dual transporter had normal eye size and retinal differentiation (Figure 8D). However, cartilage differentiation and jaw morphogenesis was disrupted. In particular, alcian blue staining was severely reduced and Meckel’s cartilage and the ethmoid plate appeared absent (Figure 9D). Fin development was not affected by mutations in this nucleotide-sugar transporter. Normal
eye and fin morphology, but similarly disrupted jaw development was observed in mutants for UDP-glucuronic acid decarboxylase.

Together these data suggest that defects in both de novo nucleotide synthesis and UDP-dependent protein glycosylation contribute to the *perplexed* phenotypes. Specifically, retinal precursor cell proliferation is likely due to reduced levels of nucleotides for DNA synthesis as both *cad* and *thymidylate synthase* mutants show reduced retinal size. The retinal lamination and differentiation phenotypes of *perplexed*, however, are more likely to be caused by pathways other that DNA synthesis because *thymidylate synthase* mutants show normal retinal lamination and cellular differentiation. The jaw defects in *perplexed* can be attributed to UDP-dependant protein glycosylation, as mutants that affect sub-sets of this post-translational modification pathway show defects in jaw development. Cumulatively, our comparison of multiple mutants suggests that the *perplexed* phenotype is complex and not simply due to a generalized decrease in de novo pyrimidine biosynthesis. Instead, the defects of *perplexed* are due to the misregulation of UDP, which impinges on several critical pathways that have tissue specific consequences.

**DISCUSSION**

The *perplexed* mutation in zebrafish affects cell proliferation and differentiation in a tissue restricted manner. Specifically, retinal, tectal, jaw and fin morphogenesis is affected. Using positional cloning techniques we have identified a mis-sense mutation within the carbamoyl phosphate synthetase 2 domain of the *cad* gene in *perplexed* embryos. Targeted gene knock-down and pyrimidine rescue experiments confirmed this mutation as causative for the *perplexed* phenotypes. Similar phenotypes between *cad* morphants (embryos in which *cad* was inhibited by anti-sense oligonucleotides) and hi2694 (a retroviral insertional mutant in the first exon of *cad*) strongly suggest the *perplexed* mutation results in loss-of-function for CAD activity. The tissue specificity of the *perplexed* mutation can be explained by the high levels of gene expression in the tissues which display dismorphic phenotypes. The *Drosophila cad* mutation and our analysis of *cad* expression in other vertebrates suggest that
there is a tissue enhanced developmental expression of this gene that is conserved through evolution.

Our analyses also suggest that the utilization of products of de novo pyrimidine biosynthesis may differ between different cell types. Comparative analysis of mutations that differentially affect either de novo nucleotide synthesis or UDP-dependant glycosylation suggests that retinal development requires de novo nucleotide synthesis for proliferation and UDP-dependant glycosylation for differentiation. Jaw development does not appear to require de novo nucleotide synthesis, but is dependent on the role of CAD in facilitating glycosylation. Although DNA/RNA synthesis and UDP-dependant glycosylation are the principal downstream pathways affected by CAD, additional uncharacterized metabolic products of CAD may also be important for the phenotypes associated with the perplexed mutation. In particular, phenotypes such as retinal differentiation, where we were unable to find a similar defect in other downstream metabolic mutants, may be due to alternative pathways. Our comparative analysis has been limited to currently identified mutations.

The imaging experiments to directly measure the cell cycle period of retinal progenitor cells are consistent with an important role for CAD in cell proliferation. Retinoblasts with the perplexed mutation required twice as long to complete one cell cycle and also showed increased cell death. One possibility for the increase in cell death might be due to a lack of pyrimidine nucleotides required for S-phase and a subsequent induction of a check point arrest, which activates apoptosis. Alternatively, activation of apoptotic pathways may be more direct as recent studies have indicated that CAD is a target for caspase-mediated degradation during cell death and loss of CAD activity, therefore, facilitates apoptosis (HUANG et al. 2002).

Observations with perplexed described here are consistent with previous studies that have implicated CAD as a key target of signaling pathways that regulate cell proliferation and differentiation (reviewed in HUANG and GRAVES, 2003). For example, in cell culture CAD was found to be a direct target of Mitogen Activated Protein (MAP) kinases. The MAP kinase Erk2 was found to phosphorylate CAD and increase its enzymatic activity
Direct phosphorylation of CAD by Protein Kinase A (PKA) was also found to correlate with increased activity (CARREY et al. 1985; CARREY et al. 1993; SIGOILLOT et al. 2002). Interestingly, PKA phosphorylation also rendered CAD more susceptible to degradation, suggesting negative feedback regulation (CARREY et al. 1986). Finally, an important role for CAD in proliferation is supported by the observation that CAD activity is upregulated in multiple cancer cell lines (KIZAKI et al. 1980; AOKI and WEBER, 1981; SIGOILLOT et al. 2004).

In addition to post-translational regulation, CAD is also regulated transcriptionally and in a manner consistent with growth and differentiation. For example, when proliferation of ts13 cells is inhibited by serum starvation, cad mRNA synthesis and stability is decreased (RAO and DAVIDSON, 1988). The opposite effects on cad mRNA are observed with serum stimulation of quiescent cells. Likewise, when cells of the HL-60 myeloid line are induced to undergo terminal differentiation, cad mRNA expression is nearly extinguished (RAO et al. 1987). Compelling evidence now implicates a direct Myc-dependent mechanism for activating the cad promoter during proliferation (MILTENBERGER et al. 1985; BOYD et al. 1997; BUSH et al. 1998). Further analysis of the cad promoter has indicated that the Brg1 complex, a SWI/SNF-type chromatin remodeling complex, associates with Myc to induce cad gene expression (PAL et al. 2003). In the absence of high levels of Myc, the Brg1 complex associates with transcriptional repressor proteins and cad transcription levels fall. Interestingly, the young mutation in zebrafish, which shows delayed cell cycle exit and blocked retinal cell differentiation, is caused by a null mutation in brg1 (LINK et al. 2000; GREGG et al. 2003). Furthermore, this mutation inhibits a wave of MAP kinase activity that normally associates with retinal cell differentiation (GREGG et al. 2003).

Overall, our analysis of the perplexed mutation indicates that the pyrimidine enzyme CAD has a central, but tissue specific role in coordinating cell proliferation and differentiation, supporting a model that was proposed based on numerous cell culture findings (HUANG and GRAVES, 2003). The comparative analysis of perplexed to zebrafish mutants that affect DNA nucleotide precursors or UDP-dependent glycosylation, suggests that the role of CAD is complex and may differ in function depending on cell type. For example within the
retina, CAD appears to be required during proliferative phases to provide sufficient quantities of DNA nucleotides for genome replication. Without CAD, the time needed to complete one cell cycle of a retinal neuroepithelial cell is extended two-fold. Retinal cell differentiation is also disrupted in *perplexed* and this phenotype sets *perplexed/CAD* mutants apart from other mutants that affect de novo synthesis of DNA nucleotides. We suggest that the role of CAD in UDP-dependant glycosylation is the reason for the retinal differentiation phenotype. Future studies to disrupt specific UDP-dependant glycoproteins will be required to identify the key substrates for retinal cell differentiation.

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REFERENCES

AMSTERDAM, A., R. M. NISSEN, Z. SUN, E. C. SWINDELL, S. FARRINGTON et al., 2004


CARREY, E. A., 1986 Nucleotide ligands protect the inter-domain regions of the multifunctional polypeptide CAD against limited proteolysis, and also stabilize the thermolabile part-reactions of the carbamoyl-phosphate synthase II domains within the CAD polypeptide. Biochem J 236: 327-335.


LINK, B., P. KAINZ, T. RYOU and J. DOWLING, 2001 The perplexed and confused mutations affect distinct stages during the transition from proliferating to post-mitotic cells within the zebrafish retina. Developmental Biology 15: 436-453.


MORFORD, G., J. N. DAVIDSON and E. C. SNOW, 1994 Appearance of CAD activity, the rate-limiting enzyme for pyrimidine biosynthesis, as B cells progress into and through the G1 stage of the cell cycle. Cell Immunol 158: 96-104.


PAL, S., R. YUN, A. DATTA, L. LACOMIS, H. ERDJUMENT-BROMAGE et al., 2003
mSin3A/histone deacetylase 2- and PRMT5-containing Brg1 complex is involved in
PATTERSON, D., R. BERGER, J. BLESKAN, D. VANNAIS and J. DAVIDSON, 1992 A single base
change at a splice acceptor site leads to a truncated CAD protein in Urd-A mutant Chinese
RAO, G. N., E. S. BUFORD and J. N. DAVIDSON, 1987 Transcriptional regulation of the human
RAO, G. N., and J. N. DAVIDSON, 1988 CAD gene expression in serum-starved and serum-
stimulated hamster cells. DNA 7: 423-432.
SHIMODA, N., E. W. KNAPIK, J. ZINITI, C. SIM, E. YAMADA et al., 1999 Zebrafish genetic map
SIGOILLOT, F. D., D. R. EVANS and H. I. GUY, 2002 Growth-dependent regulation of
mammalian pyrimidine biosynthesis by the protein kinase A and MAPK signaling
SIGOILLOT, F. D., S. M. SIGOILLOT and H. I. GUY, 2004 Breakdown of the regulatory control
SINGER, A., H. PERLMAN, Y. YAN, C. WALKER, G. CORLEY-SMITH et al., 2002 Sex-specific
STURTEVANT, A. H., 1913 The linear arrangement of six sex-linked factors in Drosophila as
<table>
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<tr>
<th>Parameter</th>
<th>wild type</th>
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<tr>
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<tr>
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Figure 1. Illustration of the role of the carbamoyl phosphate synthase aspartate transcarbamylase dihydroorotase enzyme (CAD) in de novo biosynthesis of Dihydroorotate (DHO) and its rapid conversion to Uridine diphosphate (UDP). CAD utilizes Glutamine (Gln), Adenosine triphosphate (ATP) and Bicarbonate (HCO$_3^-$) to synthesize DHO. All reactions take place in the cell cytoplasm except for that of Dihydroorotate dehydrogenase (DHO-DHase), which occurs at the mitochondria inner membrane (IM). Loss of CAD activity can affect both DNA/RNA nucleotide biosynthesis as well as UDP-dependent protein glycosylation events.
Figure 2. Positional cloning of the *perplexed* mutation. (A) Genetic and physical map of the critical interval for the *perplexed* locus of chromosome 20. Informative markers are listed with the associated number of recombination events per 1060 meioses (red). BAC, PAC and genomic scaffolds are to the right of the genetic map. The *cad* open reading frame is represented as a blue arrow. (B) Sequence analysis and predicted protein composition of wild type and the *perplexed* mutation at amino acid 1283. (C) Sequence comparison among diverse phyla show absolute conservation for M1283 (yellow highlight) and near perfect similarity for surrounding residues. Amino acids different from zebrafish are indicated in blue. The accession number for the complete coding sequence of zebrafish *cad* is AY_880246.
Figure 3. CAD knock-down phenocopies the *perplexed* mutation. (A) Whole embryo morphologies of 84hpf wild type, *perplexed* mutant, and CAD MO1 morphant embryos. (B) RT-PCR analysis of pre-mRNA splice disrupting morpholinos indicate altered mRNA processing. Control morpholino injected embryos produced the expected 288 bp product, while the splice disrupting morpholinos produced alternate splice forms. (C) Histological analysis of wild type, *perplexed*, and CAD MO1 morphant eyes. Indistinguishable phenotypes were observed in *perplexed* and CAD morphant embryos.
**Figure 4.** Rescue of *perplexed* retinal phenotype by CAD products. Retinal histology of (A) wild type embryos injected with buffer, (B) *perplexed* embryos injected with buffer, (C) *perplexed* embryos injected with uridine, and (D) *perplexed* embryos injected with orotic acid. Embryos were injected at 20 hpf and analyzed at 72 hpf by using Hoechst to stain nuclei in cryosections. Note the partial rescue of both eye size and lamination in C and D. Arrowheads indicate inner and outer plexiform lamination in A, C, and D.
Figure 5. In situ mRNA analysis of zebrafish cad. (A) Maternal stores of cad are found in embryos prior to zygotic transcription. (B) 18 somite stage embryos begin to show restricted high level mRNA expression in the eyes and anterior CNS. (C) Sagital view of a 24 hpf embryo shows cad expression has become further enhanced in the eyes and tectum. (D) Dorsal view of a 24 hpf embryo shows high cad expression within the retina, tectum and proliferative zone of the hindbrain. (E) Sagital and (F) dorsal views of 48 hpf embryos show expression in retinal and tectal proliferative zones, condensing jaw cartilage, and fin buds.
Figure 6. High levels of *cad* expression in zebrafish, chick, and mouse retinas.

Proliferative stages of retinal development were analyzed for *cad* expression in (A) zebrafish, 24 hpf; (B) chick, stage 22; (C) albino mouse, embryonic day 11. Post-mitotic stages of retinal development were also analyzed for *cad* expression in (D) zebrafish, 84 hpf (note the high level of expression in the proliferative retinal marginal zone, arrowheads); (E) chick, stage 38; (F) mouse, postnatal day 3. Asterisks denote high level of expression in the inner nuclear layer at this time. Basal surface is to the right and apical to the left. Reduced pigmentation in (D) was due to use of phenylthiourea which blocks pigment synthesis in zebrafish. Reduced pigmentation facilitated assessment of gene expression in the retinal pigment epithelium.
Figure 7. Confocal time-lapse analysis of retinal cell cycle dynamics. (A) wild type and (B) perplexed retinal progenitors. The red pseudo-colored cell expressed histoneH2B:::GFP and can be seen to enter mitosis in the upper left (time = 0:00). Representative images are shown throughout the cell cycle. Green cells, also labeled with histoneH2B:::GFP, were followed by separate analyses. Yellow numbers show time in minutes from the first imaged M-phase. The lens is located in the upper half of each image and the ventricular zone at the retinal pigment epithelium is towards the bottom (pigmentation was inhibited with PTU). Apoptosis can be seen occurring in some labeled cells in the perplexed retina (highlighted by yellow circles). Non-labeled cells dying are also visible by their pyknotic profiles.
Figure 8. Retinal histology of CAD pathway mutants. (A) wild type; (B) perplexed; (C) thymidylate synthase mutant (D) UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter mutant. Note the small eye, but normal differentiation in the thymidylate synthase mutant. No ocular defects were observed in the UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter mutant.
**Figure 9.** Jaw and fin phenotypes of CAD pathway mutants. (A) wild type; (B) *perplexed*; (C) thymidylate synthase mutant (D) UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter mutant. Alcian blue staining was used to label differentiating cartilage. Note the reduced and dismorphic jaw cartilage and fins in *perplexed*. Reduced cartilage, but normal fins were observed in the UDP-glucuronic acid/UDP-N-acetylgalactosamine dual transporter mutant. Neither jaw nor fin defects were observed in the thymidylate synthase mutants, although the eyes are small.