UNIVERSIDAD DE COSTA RICA

SISTEMA DE ESTUDIOS DE POGRADO

UNA MUTACIÓN EN UDP-GlcNac: DOLICOHOL FOSFATO N-ACETILGLUCOSAMINA-1-FOSFATO TRANSFERASA (GPT) CAUSA EL FENOTIPO *stp*^{m365} en *Danio rerio*

Tesis sometida a la consideración de la Comisión del Programa de Posgrado en *Biología* para optar por el grado de *Magíster Scientiae en Biología*

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DEDICATORIA

A mi madre y abuelos.

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Al Dr. Alejandro Leal por haber confiado en mí.

A la Dra. Ela Knapik y a todo el personal de su laboratorio en la Universidad de Vanderbilt, Nashville, Tennessee.

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PREFACIO

Generalidades del pez cebra, *Danio rerio*

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En años recientes, el pez cebra, *Danio rerio*, se ha convertido en el modelo animal por excelencia para los biólogos que estudian el desarrollo de diversos procesos biológicos. *Danio rerio*, un pequeño teleósteo de agua dulce del sur de Asia, presenta características que lo convierten en un modelo animal apropiado para la identificación de nuevos genes reguladores y el estudio de sus funciones en el desarrollo de los vertebrados. Comenzó utilizándose como modelo para la biología y la genética del desarrollo, sin embargo, se están ampliando rápidamente sus aplicaciones. Hoy en día el pez cebra se emplea en el estudio de enfermedades humanas.

Las características más importantes del pez cebra como modelo experimental que han sido la causa de su elección por muchos grupos de investigación son las siguientes:

- Fáciles de mantener y cruzar.
- Tienen un rápido desarrollo.
- Ponen cientos de huevos una vez por semana.
- Presentan desarrollo externo y los embriones son transparentes durante sus primeras horas, lo cual permite una buena observación de ellos.

- Los embriones son lo suficientemente grandes y fuertes para ser manipulados, como por ejemplo con microinyección o transplantes celulares.
- El desarrollo embrionario es sincrónico, lo que permite que todos los miembros de una misma camada estén en la misma etapa embrionaria, lo que facilita su estudio y análisis.
- Comparado con otros modelos animales vertebrados como el ratón o *Xenopus*, los órganos del embrión del pez cebra están compuestos por un menor número de células, lo que facilita su estudio. Sin embargo, los órganos operan de la misma manera que en los animales de mayor tamaño.

Además, el genoma está casi completamente secuenciado y se dispone de diversas técnicas para inhibir la expresión génica.

La embriogenésis en el pez cebra toma aproximadamente dos días, el embrión sale del corion en el tercer día de desarrollo y ya para el quinto dia, la gran mayoría de todos los tipos celulares se han diferenciado y todos los órganos corporales ya cumplen con sus funciones. Este rápido desarrollo embrionario nos permite observar todos los procesos de desarrollo y la culminación de diseños experimentales en muy poco tiempo. El pez cebra alcanza su madurez sexual entre los dos y los cuatro meses aproximadamente y su desarrollo embrionario es muy similar al de los vertebrados superiores, incluyendo al humano y otros mamíferos.

Origen del mutante *stumpf*

La manera más efectiva de inducir pequeñas lesiones intragénicas en un genoma es a través de la mutagénesis química y en el caso del pez cebra se ha utilizado el mutágeno 1-etil-1-nitrosourea (ENU, por sus siglas en inglés). ENU no ocurre en la naturaleza. Fue sintetizado por primera vez en 1919 a través de la reacción de N-etilurea con el ácido nitroso (Werner 1919). En su forma pura es un cristal amarillento que reacciona a la humedad y a la luz. Actúa por medio de etilación y carboamilación. Su grupo etilo se transfiere a sitios nucleofílicos, mientras que el grupo carbonilo se transfiere a los grupos amino de las proteínas. ENU induce la alquilación en la posición O^6 de la guanina y en la posición O^4 de la timina en el ADN. *In vitro*, la mayoría de las mutaciones inducidas por ENU son transiciones GC a AT, y en menos frecuencia las transiciones AT a GC. En *Danio rerio* muchos tipos de alteraciones en su secuencia de ADN han sido observadas, con una razón de 1:1 de sitios A/T y G/C (Knapik 2000).

Las mutaciones causadas por ENU no son fáciles de detectar y debe utilizarse para dicha detección el método de clonaje posicional. Esta metodología se basa en la identificación de individuos en familias en las cuales el rasgo fenotípico de la mutación está segregándose. Tomando el ADN tanto de miembros afectados como de miembros sanos se examinan marcadores genéticos distribuidos en todos los cromosomas, hasta encontrar uno que se detecte particularmente en aquellos individuos que estén afectados. Seguidamente, se analiza este intervalo en el genoma y se escogen genes que representen candidatos potenciales para la enfermedad y se investiga a nivel de la secuencia, si existen mutaciones. De esta manera no hay que saber de antemano qué clase de gen era el que se tenía que buscar. Esta estrategia es poderosa pero a la vez

complicada, pues se requiere en muchos casos estudiar un número grande de marcadores genéticos en todos los individuos antes de poder determinar cuál región del genoma está asociada con esta enfermedad y contiene la mutación de interés .Gracias al gran número de meiosis generado por el pez cebra, el intervalo genético que contiene la mutación puede ser reducido hasta un clon BAC.

El mutante *stumpf* (*stp*) del pez cebra tiene una mutación letal embrionaria heredada recesivamente que fue inducida por ENU en 1996 (Neuhauss *et al.* 1996). De ésta mutagénesis a gran escala fueron identificadas 48 mutaciones que afectaban el desarrollo craneofacial en *Danio rerio*, siendo *stp* una de ellas. Después del mapeo genético de la mutación y de secuenciar la región crítica por parte de un grupo de investigadores en Alemania (Schmidt, Leal y Reis 2002, sin publicar), se identificó una mutación puntual que resulta en el cambio de un aminoácido en la posición 187 (G187D) de la UDP-GlcNAc:dolicol fosfato N-acetilglucosamina-1-fosfato transferasa (GPT, por sus siglas en inglés). El residuo de glicina en ésta posición es altamente conservada en todos los filos eucarióticos, y en contraste con la glicina, el aspartato es un amino ácido altamente acídico.

GPT es la primera enzima que cataliza el primer paso del ciclo del dolicohol, vía necesaria por la cual se ensambla el oligosacárido ligado a lípidos, dol-PP-$GlcNAc_2Man_9Glu_3$, esencial para la N-glicosilación de proteínas. La correcta N-glicosilación de las proteínas es un prerrequisito para asegurar su correcto plegamiento en el retículo endoplasmático y su transporte hacia el aparato de Golgi. Errores en este proceso junto con condiciones ambientales específicas pueden causar imperfecciones en el desarrollo y conllevar a defectos congénitos o desordenes en estadios tempranos (Haltiwanger and Lowe, 2004).

Se cree que es ésta mutación en GPT la causante del fenotipo *stp*, para lo cual en 2005 se realizaron varios experimentos para probar dicha hipótesis.

Diseños experimentales

Los experimentos mencionados anteriormente que se realizaron para caracterizar y comprobar que la mutación antes descrita es la causante del fenotipo *stp* se detallan a continuación:

- Uso de tinciones específicas para determinar y visualizar niveles de N-glicosilación de proteínas tanto en el mutante como en el silvestre.
- Métodos para determinar y visualizar defectos a nivel celular.
- Métodos para determinar y visualizar los efectos de la pérdida de función del gen en cuestión.
- Métodos de bloqueo químico de la proteína codificada por el gen.

Los resultados de estos experimentos indican que la proteína GPT está involucrada en el desarrollo craneofacial del pez cebra. El mapeo genético determinó la existencia de una mutación puntual en el gen *Gpt*. Tomando esto como partida, el uso de tinciones especificas para visualizar defectos en la N-glicosilación (ensayo de aglutinina de gérmen de trigo) indicó un déficit de proteínas glicosiladas craneofaciales y bajos niveles de N-glicosilación en las proteínas del mutante, en comparación con los individuos silvestres. Las técnicas moleculares utilizadas para evaluar los efectos de pérdida de función del gen (microinyección de morfolinos), indican que la pérdida de

función de GPT en un embrión de pez silvestre fenocopia al mutante, es decir, el fenotipo resultante de dicha inyección es muy similar al que se manifiesta en el mutante. Los resultados obtenidos con el uso de tunicamicina para bloquear químicamente la N-glicosilación en individuos sanos, son otra evidencia de que la mutación en *Gpt* genera el fenotipo mutante *stp*. Por medio de este método los individuos silvestres presentan defectos en la N-glicosilación de sus proteínas craneofaciales, tal y como se observa en el mutante. Este trabajo es el primero en utilizar al pez cebra como modelo animal para estudiar la importancia de la N-glicosilación de las proteínas en el desarrollo craneofacial de los vertebrados.

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A Mutation in UDP-GlcNAc:Dolichol Phosphate N-Acetylglucosamine-1-Phosphate Transferase (GPT) causes the *stp*^{m365} phenotype in *Danio rerio.*

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ABSTRACT: *In this work we show that the zebrafish* stp^{m365} *mutation interferes with the craniofacial development by disrupting the gene that encodes for the* Gpt *enzyme which is involved in the first critical step of the N-glycosylation pathway.*

Key words: zebrafish, craniofacial development, n-glycosylation, stp^{m365}

Introduction

A large-scale ENU mutagenesis screen (Driever *et al.* 1996) identified 48 mutations affecting craniofacial development in zebrafish (Neuhauss *et al.* 1996). Complementation analysis showed that these mutations correspond to 34 genetic loci. The *stumpf* ^{m365} (*stp* ^{m365}) mutation was isolated as one of the 48 mutations with abnormal craniofacial development in the ENU screen (Neuhauss *et al.* 1996).

The pharyngeal skeleton of all vertebrates is derived from the neural crest cell (NCC) population. NCCs originate at the neural plate border and are located at the dorsolateral aspect of the neural tube prior to migration. In *Danio rerio*, anterior cranial neural crest cells start to emigrate at intermediate segmentation stages (~ 15 hours post fertilization or hpf), followed by cells from progressively more caudal origins. These cells migrate ventrally and rostrally and subsequently form a reiterated series of seven pharyngeal arch primordia separated by endodermal epithelial pouches. By 72 hpf, many of the neural crest derived mesenchymal cells have differentiated into long rows of chondroblasts within each of the seven pharyngeal arches (Schilling and Kimmel 1994). Following the establishment of the pharyngeal arches, chondrogenic neural crest cells begin to aggregate. These mesenchymal condensations form the rough outline of the future skeletal elements. Subsequently, the condensed cells will divide, secrete an extracellular matrix (ECM) and differentiate into chondrocytes (Yan *et al.* 2002, Neuhauss *et al.* 1996). This ECM is a complex structure that is built from a large number of different components. These include proteins (collagens, fibrillin and fibronectin among others) and various

glyco-conjugates. The ECM plays an important role in numerous biological processes during development (Krieg and LeRoy 1998).

Many of these extracellular matrix proteins are modified by glycosylation. Almost all eukaryotic cells produce proteoglycans and either secrete them into the extracellular matrix, insert them into the plasma membrane, or store them in secretory granules (Haltiwanger and Lowe 2004).

The posttranslational modification of proteins with asparagine (N)- linked oligosaccharides (N- glycosylation) is a universal feature of eukaryotic cells. It is well established that N-linked sugar chains at the cell surface participate in cell-cell recognition and intercelular communication during development. Suboptimal N-glycosylation is likely to impair some of these functions (Wu et al 2003).

The earliest steps in N- glycan formation are conserved from yeast to humans with the production of the dolichol oligosaccharide precursor. This stucture is synthesized in a stepwise manner beginning with the UDP-GlcNAc: dolichol phosphate N-acetylglucosamine-1-phosphate transferase (GPT) (Marek *et al.* 1999). The function of GPT is to catalyze the transfer of Glc-Nac-1-P from UDP- GlcNac to Dolichol-phosphate (Dol-P) to form GlcNAc-P-P-Dol. The GPT gene is highly conserved among various eukaryotic organisms (Zhu and Lehrman, 1990) to the extent that the human gene encoding GPT can be isolated by complementation of the yeast homologue ALG7 (Ekert *et al.* 1998). The structure of dolichol oligosaccharide precursor found in all eukaryotic cells indicates that GlcNAc-1-phosphatetransferase activity should be essential for precursor biosynthesis and thus for cellular N-glycosylation (Marek *et al.* 1998).

After mapping of stp^{m365} mutation and sequencing the critical genomic region, we detected a point mutation causing an amino acid exchange at position 187 (Gly to Asp) in the zebrafish homologue of UDP-GlcNAc:Dolichol Phosphate N-Acetylglucosamine-1-Phosphate Transferase (GPT). The lesion is a single base change as expected for ENU-induced mutations. The glycine residue in this position is conserved in all major eukaryotic phyla as exemplified by the species *A. thaliana, S. cerevisae, D. melanogaster* and *H. sapiens*. In contrast to glycine, aspartate is a highly acidic residue and this amino acid appears to be located in the active domain of the GPT enzyme.

Here we present evidence that the G187D point mutation in GPT is the cause of the *stp* phenotype.

Materials and Methods

Fish husbandry

Fish were raised and kept under standard laboratory conditions at 28.5°C (Westerfield 1995). Embryos were staged and fixed at specific days post fertilization (dpf) as described by Kimmel *et al.* in 1995. To inhibit pigment formation and thus visualize internal structures in some experiments, embryos were grown in 1X PTU (1-phenyl-2-thiourea) (Sigma). The *stp*^{m365} mutation was identified in a screen by Neuhauss *et al.* in 1996.

Cartilage staining

Alcian Blue staining was used to visualize the cartilage skeleton of the zebrafish with a protocol modified from Neuhauss *et al.* (1996). Embryos at 4 dpf were anesthetized with 0.02% Tricaine (Sigma) and fixed overnight at 4°C in 4% paraformaldeyde (PFA). Following two washes of ten minutes with PBS (phosphate-buffered saline), embryos were bleached in 1.3 ml of 10% hydrogen peroxide (H_2O_2) with 100 µl of 1M KOH for an hour and forty minutes. They were then stained overnight at room temperature in 0.1% Alcian Blue dissolved in acidic ethanol (70% ethanol, 5% concentrated hydrochloric acid), washed overnight in acidic ethanol, dehydrated with 85% and 100% ethanol followed by storage at 4° C in 80% glycerol.

Immunochemistry

For visualizing specific defects in the levels of glycosylation in the mutant as compared to the wild type phenotype, the Wheat Germ Agglutinin Assay was used. Embryos were grown in 0.2 mM PTU in eggwater and fixed at the required stages in 4% PFA at 4°C overnight. The next day the embryos were washed with PBT (PBS – 0.1% Tween 20) and brought stepwise (25%-50%-75%) to 100% MeOH. Embryos were then rehydrated to PBT stepwise. At 4 dpf embryos were digested with Proteinase K (50ug/ml) for 1 hour at room temperature Following the digestion, embryos were washed with PBT and fixed with 4% PFA for 20 minutes at room temperature. Following extensive washes with PBT, the embryos were incubated overnight at 4° C with Biotinylated Wheat Germ Agglutinin (5mg/ml, Vector Laboratories) diluted 1:10000 in PTD (0.3% Triton X-100 + 1% DMSO in PBT) .The next day the embryos were washed with PTD and incubated in ABC complex (Vector Lab) for 1 hour at room temperature. The embryos were then incubated for 10 minutes with DAB detection kit or until staining was achieved. Embryos were washed with PBT and stored at 4°C in 80% glycerol.

Genetic mapping and cloning

The positional cloning strategy was used to identify the gene responsible for the *stp* phenotype (Knapik *et al.*1996). Genetic and physical mapping was employed to detect a mutation in the zebrafish homologue of the GPT gene.

The *stp* heterozygote fish was mated to a polymorphic background wild-type fish and the F1 family was raised. The F1 family was intercrossed to identify heterozygote individuals that carry the mutant allele. These heterozygote pairs were used to generate homozygous mutant embryos (F2).

The DNA from F2 individuals was used as a template in a total genome scan using bulk segregant analysis. Specifically, pools of wild-type and mutant embryo DNAs were screened by PCR for simple sequence length polymorphism (SSLP) markers linked to the mutation. Once linkage was found, the precise position of the mutation on the chromosome was determined by genotyping all the markers in this region, and a local order was established by ordering recombinations in 3292 meioses.

The gap between the closest SSLP markers and the mutation was closed by identifying overlapping PAC clones that covered the interval. The Single Strand Conformation Polymorphism (SSCP) markers derived from the PAC clones were used to further narrow the critical interval until the genomic sequence between the last two recombinants flanking the mutation point was reduced to about 50 kb. This region was screened and candidate genes were identified using the NCBI sequence database, the Zebrafish Genome Sequencing Project (Sanger Center) and the gene prediction program NIX.

Whole-mount *in situ* hybridization

Embryos where collected at specific developmental stages and processed for whole-mount *in situ* hybridization as previously described by Thisse *et al.* (1993). Digoxygenin-labeled riboprobes were synthesized from templates linearized with *EcoR1* using T7 RNA polymerase for *col IX* (provided by Dr. Iain Drummond) and *col2α1* (Yan *et al.* 1995), *Hin*dIII with T3 RNA Polymerase for *hsp47* (Pearson *et al.* 1996) and *EcoRV* using T7 RNA polymerase for *sox9a*.

Cloning of GPT (*stp* ^{m365})

An RT-PCR product was purified with QUIAquick PCR Purification Kit (Qiagen) according to manufacturers instructions and digested with BamHI and XhoI restriction enzymes (Fermentas, MBI). GPT was ligated to the expression vector pCS2+ cut with the corresponding enzyme.

Sequence of the cDNA was verified by Sanger sequencing.

Messenger RNA injection for rescue experiment

The *gpt* wild type gene was cloned in pCS2+ vector. Capped *gpt* mRNA was synthesized using the mMessage mMachine kit (Ambion) and injected at a concentration of 20 pg. 1 ul of diluted mRNA was injected into one to two-cell embryos using a gas-driven microinjector (WPI, PV820 Pneumatic PicoPump). Capillaries for injections were pulled on a Sutter Instruments (P-97) pipette puller.

***zgpt* antisense oligo injections**

The antisense oligonucleotide used was a 25-mer morpholino oligo (Gene Tools, LLC) with the following sequence:

5'-CAACAGGAATTGGAGACATCTTCTC-*3'*

The oligo was resuspended in RNase-free water (Fisher) and diluted to working concentrations ranging from 1ng to 5.3 ng. 1 ul of morpholino dilution was injected into one to two-cell embryos using a gas-driven microinjector (WPI, PV820 Pneumatic PicoPump). Capillaries for injection were pulled on a Sutter Instruments (P-97) pipette puller. Morpholino-treated embryos are referred to as "morphants".

Tunicamycin assay

Tunicamycin (TU) is a steric analog of the Gpt substrate UDP-GlcNAc and thus acts as competitive inhibitor of the GPT enzymatic function. To ablate the function of the Gpt protein, wild type embryos were treated with non-lethal dosis of Tunicamycin (5mg/ml; Sigma) from day 2 to day 4 post fertilization. During incubation, embryos were monitored and then fixed in 4% PFA. Wheat Germ Agglutinin Assay was performed on these embryos for further analysis.

RESULTS

stp^{m365} MUTANTS SHOW DEFECTS IN CRANIOFACIAL CARTILAGE DIFFERENTIATION AND MORPHOLOGY.

The zebrafish *stp*^{m365} allele was isolated in a large–scale ENU-mutagenesis screen for craniofacial development defects (Driever 1996, Neuhauss *et al.* 1996). The *stp*^{m365} mutation is inherited in a recessive way and the mutant phenotype segregates as Mendel law predicts (25% mutants). The mutant embryos are characterized by a small reduction of the body length, small eyes/lenses, short head, small and kinked pectoral fins as well as thinner and

malformed cartilages (Figure 1). The phenotype can be observed around 4 dpf. The embryos also present a de-inflated swim bladder and die around 6-8 dpf, probably due to inability of the embryos to swim and gather food.

We stained head cartilages of the mutant 4 dpf embryos with Alcian Blue dye to visualize the cartilaginous craniofacial skeleton. Analysis of the Alcian Blue stained embryo reveals a very specific malformation in the ceratohyals: they are oriented perpendicularly to the embryonic anteriposterior axis (Figure 1).

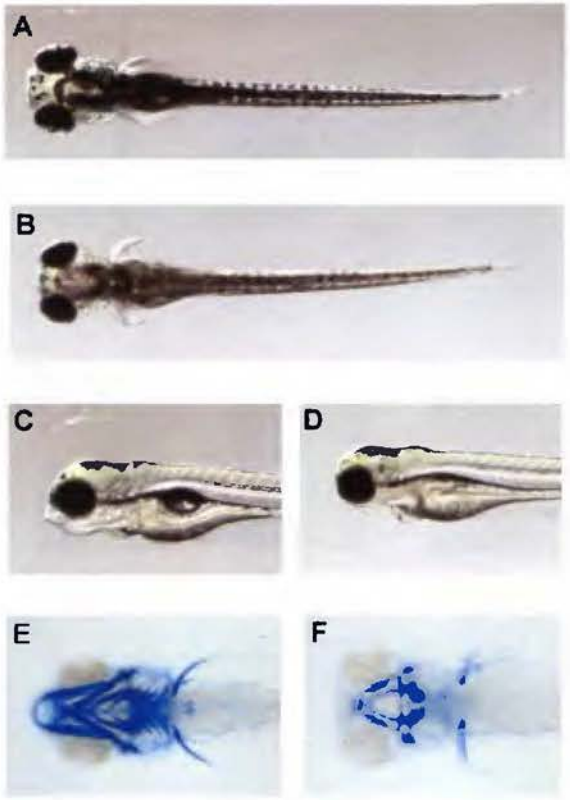

Figure 1: Morphological wild type and *stumpf* phenotype and Alcian Blue cartilage preparations. Live wild type (A, C) and *stumpf* (B, D) fish appearance at 4 dpf and wildtype (E) and *stumpf* (F) head skeleton. The cartilages in the *stumpf* mutant are smaller and malformed (F). Arrows show the misplaced ceratohyals in the mutant as compared to the wild type.

MOLECULAR NATURE OF THE *stp*^{m385} MUTATION.

Genotyping of wild-type and mutant pools of DNA from F2 animals with SSLP markers (Knapik et. al. 1998) linked *stp* ^{m385} to LG5. Linkage was confirmed by genotyping of individual F2 mutant embryos with the flanking markers Z20915 and Z25627. Using over 3200 meioses we established a fine map of the region and were able to restrict the critical genetic

interval harboring the *stp* ^{m365} mutation between markers Z25039 (3 recombinants out of 3292 meioses) and Z9419 (3 recombinants out of 2618 meioses) (Figure 2). The genetic distance between these markers is 0.17 cM. The last two recombinants enclosed a genomic region of about 40 kb length containing the locus of the *stumpf* mutation. By using annotation programs only two EST's codify in this region. Both were sequenced in wild type, heterocygote and mutation embryos. Only the gene coding for the zebrafish homologue of *gpt* showed a point mutation (G560A). The *stp* mutation converts a glycine to an aspartate (G187D) (Figure 2). Sequence comparisons between different organisms revealed a high degree of evolutionary conservation.

To confirm that reduction in *gpt* function results in the *stp* phenotype, we injected embryos with morpholino antisense oligonucleotides targeted to *gpt*. Injecting homozygous wild type embryos with 5.2 ng of MO resulted in a *stp*^{m365} mutation phenocopy (Figure 3 and 4).

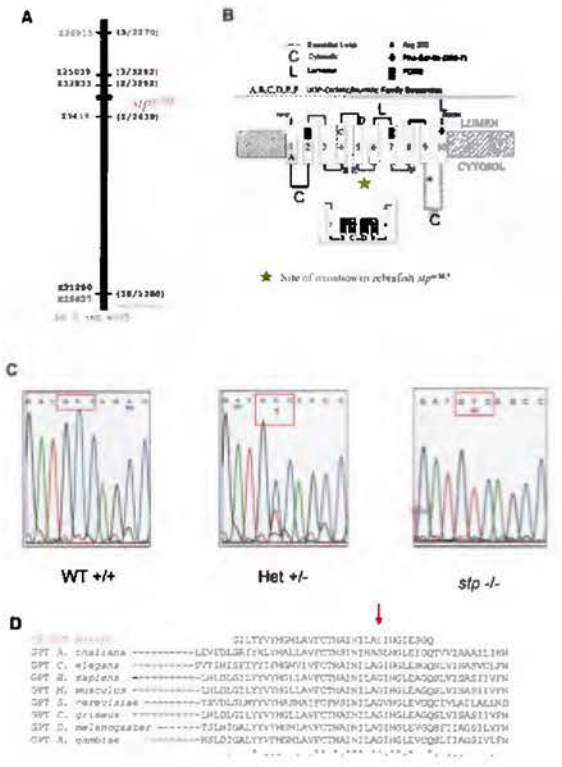

Figure 2: Cloning of the *stp*^{m365} gene. (A) The *stp*^{m365} mutation maps to zebrafish chromosome 5 (LG 5). (B) Diagram of the Gpt protein and the site of mutation in zebrafish. (C) Electropherograms of wildtype +/ +, heterozygous +/- and *stp*^{m365} -/- fish. The G to C transversion results in an amino acid exchange (G560D). (D) The glycine residue is conserved in all phyla from organisms like *A. thaliana*, *S. cerevisae*, *D. melanogaster* to *H. sapiens*.

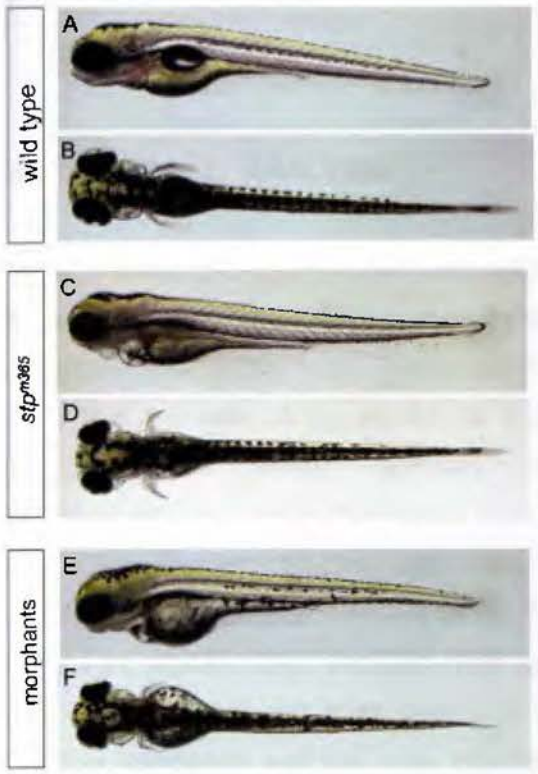

Figure 3: Knockdown of the *gpt* gene. Injection of *gpt* antisense morpholinos (5.2 ng) to wild type embryos results in *stp*^{m365} phenocopy. *gpt* morphants (E, F) are morphologically indistinguishable from *stp*^{m365} mutant embryos (C, D) at 4dpf, displaying reduced body length, smaller head and lenses and kinked pectoral fins.

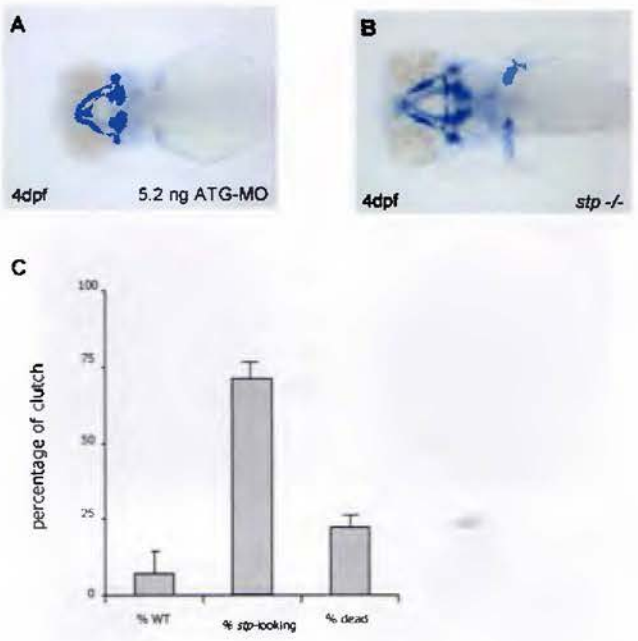

Figure 4: *gpt* morphant head cartilages stained with Alcian Blue (A) are indistinguishable from *stumpf* preparations (B). This result is consistent in nearly 75% of the injected clutches (C).

HISTOLOGICAL ANALYSIS.

Plastic sections of the head region were performed to analyze the mutant defects at the cellular level. These sections show that the cells of the *stp* fish at 4 dpf are poor in shape, specially the chondrocytes and the photoreceptor cells of the retina, known for its high rate of glycosylation (Figure 5). The eye of *stp* ^{m365} mutant is smaller than the wild-type eye. The photoreceptor cell layer seems to be missing completely and the outer plexiform layer and marginal zone are not clearly defined. The cartilages are smaller and fail to make the "stack of coins" pattern. The *stp* extracellular matrix does not show the typical metachromatic color change with toluidine blue staining (Figure 5). This suggests that the composition of the extracellular matrix is altered.

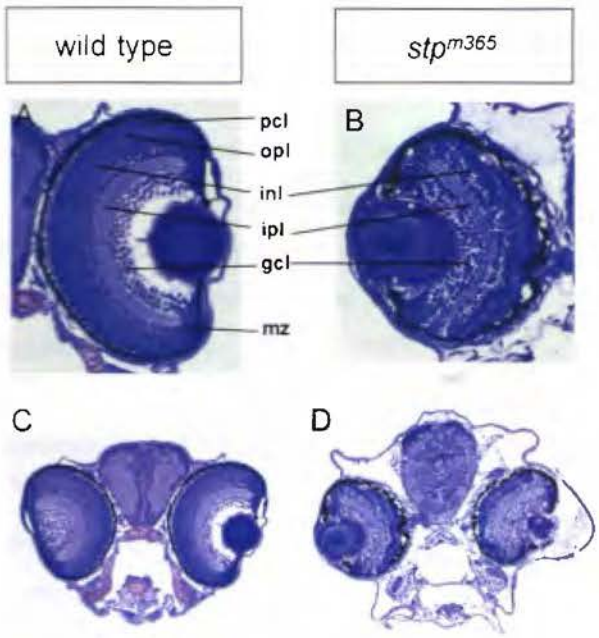

Figure 5: Toluidine Blue staining of 5µm transverse plastic sections of wildtype (A) and *stp*^{m365} (B) eyes. The eyes of the *stp*^{m365} mutants are smaller. Some layers are missing while others can not be clearly distiunguished. Abbreviations: pcl, photoreceptor cell layer; opl, outer plexiform layer; inl, inner plexiform layer; lpl, inner plexiform layer; gcl, ganglion cell layer; mz, marginal zone.Histological analysis of craniofacial chondrocytes of 4dpf wildtype (C) and stp^{m365} embryos (D). The loss of *gpt* activity in the mutant embryos leads to absence of the metachromatic staining of cartilage extracellular matrix.

stp^{m365} MUTANTS SHOW ABNORMAL LEVELS OF N-GLYCOSYLATION IN THEIR CRANIOFACIAL SKELETON.

To compare levels of N-glycosylation on *stp* ^{m365} mutants and *gpt* morphants wholemount stainings with the lectin Wheat Germ Agglutinin (WGA) were performed (Figure 6). In 4 dpf wild-type embryos the craniofacial skeleton shows a very strong WGA staining, indicating normal levels of N-glycosylation, whereas *stp*^{m365} embryos show a severe reduction of the WGA staining. This indicates a disruption in the N-glycosylation pathway in the affected embryos.

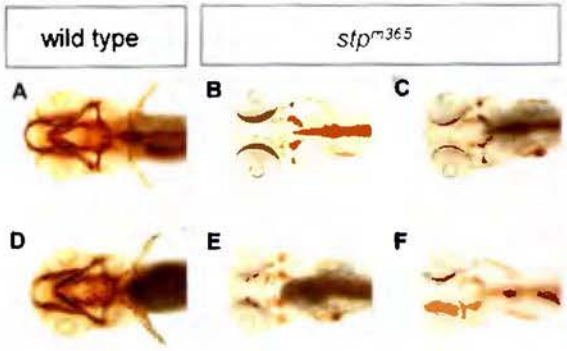

Figure 6: Wheat Germ Agglutinin (WGA) labeling of glycosylated proteins in wild type and *stumpf* siblings at 4 dpf. WGA binds to sialic acid and N-acetylglucosaminyl residues of proteoglycans in wild type dorsal neurocranium (A) and viserocranium (D) chondrocytes, while a severe reduction of WGA binding is revealed in *stumpf* craniofacial skeleton (B, C, E,F).

MOLECULAR ANALYSIS OF THE EARLY *stp*^{m365} PHENOTYPE.

The *col2α1* gene (encoding type II collagen alpha chain 1) is expressed in precartilage condensations and differentiating cartilage within the zebrafish cranium and it is a necessary component of the cartilage differentiation pathway (Yan *et al.* 1995). Its expression at 2 and 3 dpf is normal in *stp*^{m365} mutants, indicating that the chondrogenic pathway is not affected. Another collagen gene, *collX alpha1* (encoding type IX collagen alpha chain 1) is expressed in the otic vesicle at 2 dpf. The *stp*^{m365} mutants also display a normal expression of this gene at 2 and 3 dpf (Figure 7).

Interestingly, a distinct expression pattern of another marker is seen in the mutants as compared to the wild type embryos. *sox9a* is a duplicate ortholog of the human *SOX9* gene and it is suggested that the zebrafish gene or its downstream targets, perhaps including extracellular matrix proteins, play morphogenetic roles in chondrogenesis. It is known that *sox9a* is essential for the formation of cartilages in the neurocranium, pharyngeal arches and pectoral appendages (Yan et. al. 2002). The *stp*^{m365} mutant embryos display strong *sox9a* expression at 3 dpf as compared to the wild type embryos. *sox9a* expression is

upregulated in craniofacial chondrocytes in *stp*^{m365} mutant embryos at 3 dpf.

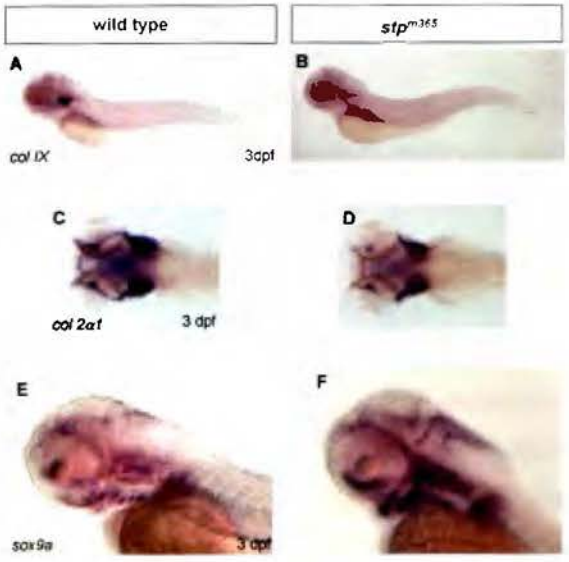

Figure 7: mRNA expression levels detected by riboprobes in *stumpf* and wild type siblings. (B, D) At 72hpf, *col2alpha1* and *collX* expression in ***stumpf*** mutants is downregulated in craniofacial chondrocytes as compared to wild type siblings (A,C). (F) ***sox9a*** transcripts in *stumpf* ^{m365} mutants at 72hpf are upregulated, as compared to wild type siblings (E).

THE WILD-TYPE GPT PROTEIN IS ABLE TO RESTORE NORMAL CRANIOFACIAL DEVELOPMENT IN *STUMPF* EMBRYOS.

To prove whether the G to D substitution in the Gpt is the SNP responsible for the *stp* phenotype, injection of Gpt wild-type mRNA was performed.

The injection of 20 pg of Gpt wild-type mRNA to 1-2 cell stage embryos from a cross between two heterozygous *stp*^{m365} carriers resulted in partially rescued 4 dpf *stp*^{m365} mutant embryos. The *stp*^{m365} injected embryos with the wild type mRNA show a less severe phenotype: clear pharyngeal arches (Figure 8C) which are not seen in the mutant (Figure 8A) as well as a less malformed ethmoid plate (Figure 8D) as compared to the mutant (Figure 8B).

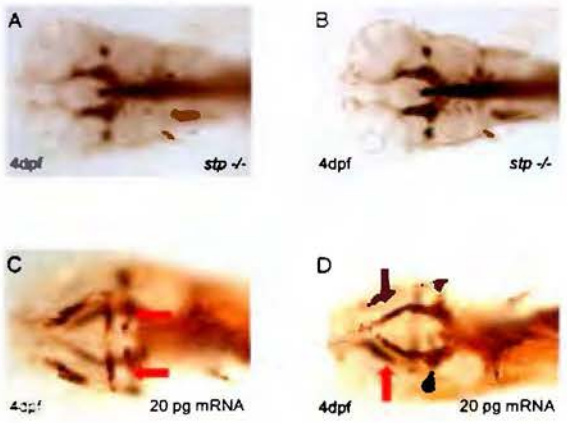

Figure 8: Rescue of the *stumpf* mutant phenotype with 20 pg of *gpt*-mRNA. (A,B) *stumpf* embryos at 4dpf present severe reduction of WGA binding in the craniofacial skeleton, suggesting a low level of N-glycosylation in the mutants. (C,D) The injection of 20 pg of *gpt*-mRNA to *stp* ^{m365} embryos rescues the mutant phenotype, as shown by an increase of the WGA binding. (C) Arrows show the stronger staining in the pharyngeal arches and ethmoid plate (D).

CHEMICAL BLOCKING OF GPT PROTEIN FUNCTION IN WILD-TYPE EMBRYOS PRODUCES A *STP*^{m365} MUTANT PHENOCOPY.

To further confirm the nature of the stp^{m365} mutation, we treated embryos with Tunicamycin (TU), a steric analog of the GPT substrate UDP-GlcNAc that acts as a competitive inhibitor of the GPT enzymatic function. The exposure of 24 hpf wild-type embryos to TU (1ug TU/ml eggwater) produces a phenocopy of the *stp*^{m365} mutant as shown by Alcian Blue staining and Wheat Germ Agglutinin Assay (Figure 9). The chemical blocking of the GPT protein by exposure of wild type embryos with TU generates 4 dpf fish with a malformed jaw (Figure 9A) similar to the jaw malformation in the mutant (Figure 9E), smaller and malformed ceratohyals (Figure 9B) similar to the phenotype observed in the mutant, are found to be perpendicular to the body axis. The mutant presents an incomplete neurocranium (Figure 9G) as well as incomplete, not stained viscerocranium (Figure 9H). These similar features can also be observed in the treated embryo (Figures 9C and 9D). This experiment confirms that the mutation in *gpt* is linked to the *stp*^{m365} phenotype.

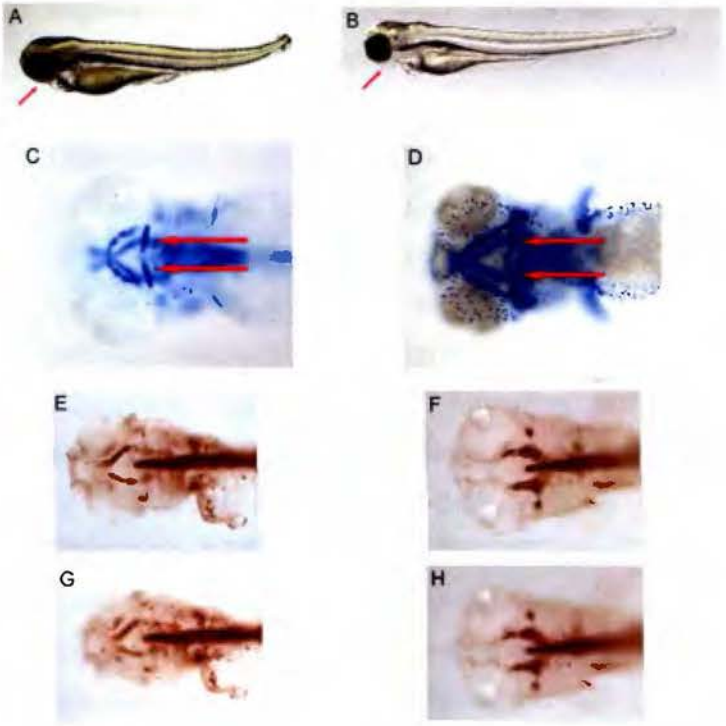

Figure 9: Chemical blocking of Gpt enzymatic activity in wild type embryos by exposure to Tunicamycin (TU).(A, C, E, G) 24 hpf wild type embryos treated with 1μg/ml TU show malformed jaw (A), misplaced ceratohyals (C) and incomplete neurocranium and viserocranium (E, G) very similar to mutant embryos (B,D,F,H).

DISCUSSION

Using diverse molecular tools we show that the zebrafish *stp*^{m365} mutation interferes with craniofacial development and morphogenesis by disrupting the gene encoding the Gpt enzyme which mediates the first critical step of the N-glycosylation pathway. Here we report that this mutation leads to several phenotypic features. Specifically, the mutant displays a reduction in body length, reduction and malformation of the eye, shortened head, thinner and shorter head cartilages (with the ceratohyals pointing to the midline) as well as malformed pectoral fins.

Evidence for the involvement of Gpt in the *stp*^{m365} phenotype is based on several findings: (1) positional cloning results; (2) *stp*^{m365} mutants display abnormal levels of N- glycosylation; (3) at 4 dpf in *stp*^{m365} fish the chondrocytes and the photoreceptor cells of the retina, known for its high rate of glycosylation, are disorganized and display differentiation defects; (4) MO antisense oligonucleotide mediated knockdown of the *gpt* gene results in a *stp*^{m365} mutation phenocopy; (5) *stp*^{m365} mutant embryos injected with wild-type *gpt* mRNA show a less severe phenotype which is clear evidence of a partial rescue; and (6) chemical blocking of Gpt protein function in wild-type embryos produces a *stp*^{m365} mutant phenocopy.

In mice, GPT is encoded by the *dpagt1* gene on chromosome 17. It is known that without GPT activity, subsequent steps necessary for constructing the oligosaccharide precursor essential for the production of asparagine – N – linked oligosaccharides (N- Glycans) in eukaryotic cells cannot occur (Lehrman *et al.* 1988). Inhibition of this enzyme using tunicamycin produces a deficiency in N-glycosylation in cell lines and embryonic lethality during preimplantation development in vivo, suggesting that N- glycan formation is essential for early embryogenesis (Marek *et al.* 1999). It was shown that embryos homozygous for a deletion in the GPT gene complete pre-implantation development and also implant in the uterine development, but die between days 4-5 postferilization with cell degeneration apparent among both embryonic and extraembryonic cell types (Marek *et al.* 1999). These results indicate that GPT function is essential in early embryogenesis and suggest that N-gylcosylation is needed for the viability of cells comprising the pre-implantation stage embryo. Pre-implantation defects have not been observed in zebrafish, which suggests that the function of GPT differs in mouse and zebrafish. N-glycosylation plays an important role in protein folding and is crucial for trafficking of proteins through the

secretory pathway. Transgenic mouse models show that correct processing of N-glycans is a prerequisite for normal embryogenesis (Marek *et al.* 1999). N-glycosylation plays an important role in protein folding and quality control in the ER (Sousa *et al.* 1992; Hammond and Helenius. 1995). One of the most important functions of N- glycans is that they are needed for the correct folding of polypetides by chaperones in the ER. Correct folding permits oligomer formation and transport of proteins to their final destination; incorrect folding targets proteins for degradation by the quality control system in the ER (Hammond and Helenius. 1995; Kopito 1997). The advantages of using the zebrafish model to study the role of N- glycosylation are evident since they acquire a much higher level of organization in comparison to that of the mice, allowing the use of different tools to study their development for a longer period of time before they die.

In the hamster, GPT is encoded by ALG7, an early growth response gene, whose expression has been shown to affect the extent of N-glycosylation and secretion of proteins. Using the postnatally developing hamster submandibular gland as en experimental tissue it was shown that the expression of ALG7 might be required for normal development and differentiation (Mota *et al.* 1994). It is also proven that ALG7 expression is tissue specific, most likely reflecting different N- glycosylation capacities of specialized tissues (Mota *et al.* 1994).

In yeast, GPT was shown to be essential for cell growth, since a null mutation of GPT is lethal (Kukuruzinska *et al.* 1987). It is also known that a mutant with a diminished GPT activity had a distinctly severe phenotype, suggesting that GPT activity affects various functions in the yeast life cycle (Kukuruzinska and Lennon, 1995). Glycosylation is necessary for the progression through the cell cycle, as the *alg1* mutant arrests at the G1 phase of the cell cycle. A similar effect is seen after treatment of cells with tunicamycin (Kukuruzinska *et al.* 1987).

In frogs, intraocular injection of tunicamycin results in retinal abnormalities similar to those seen in some retinal distrophies exhibiting destruction of the photoreceptor cells (Chambers *et al.*1986). In addition to anatomic abnormalities in retinal rod outer segments, tunicamycin injection also resulted in decreased electroretinogram responses leading to blindness (Chambers *et al.* 1986). A possible involvement of the N-glycosylation in the visual process is also indicated by studies with isolated frog retinas treated with tunicamycin. Proper glycosylation seems to be required for the normal assembly of photoreceptor cells. These defects in frog retinas resemble ocular defects in the

stp^{m365} mutant fish. The eye of *stp*^{m365} mutants is smaller compared to wild - type siblings and displays retinal defects. Specifically, the photoreceptor cell layer seems to be missing completely and the outer plexiform layer and marginal zone are not clearly defined.

In humans, DPAGT1 encodes for the GPT enzyme that catalyzes the first step in the N- glycosylation pathway. Wu *et al.* 2003 described a patient with a mutation in the DPAGT1 gene causing a novel congenital disorder of glycosylation (CDG) type. CDG-Ij. The patient presents severe hypotonia, medically intractable seizures, mental retardation, microcephalia, arched palate, micrognathia and exotropia (a type of strabismus). Some of the aspects of the human mutation resemble the zebrafish *stp*^{m365} phenotype, such as microcephalia, micrognathia and optical defects. This makes the *stp*^{m365} mutant the first convenient and effective model system to study the effects of glycosylation on organogenesis. Up to now, the craniofacial malformation is the most conspicuous defect observed in *stp*^{m365} Being the role of this protein so important, it could be possible that there are other defects that can only be observed with future analyses, such as body sections of the zebrafish, this to unveil any organ defect.

There are no other *gpt* homologues in the zebrafish which might have a redundant function in the zebrafish, accounting for the mild phenotype observed in the *stp*^{m365} fish. We conclude that the early steps of N –glycosylation are required for normal chondrocyte differentiation due to the fact that this process seems to be arrested with the mutation in *gpt*, causing the *stp*^{m365} phenotype. Due to the fact that the pharyngeal arches are present but very malformed, future analyses should be done to determine the enzymatic activity of Gpt left in the *stp*^{m365} mutant. Studies targeted on the distinct expression pattern of *sox9a* seen on *Danio rerio* can bring more light on to why this gene is overexpressed in the mutant, and thus, its function in chondrogenesis processes.

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