Fishing the Molecular Bases of Treacher Collins Syndrome

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Abstract

Treacher Collins syndrome (TCS) is an autosomal dominant disorder of craniofacial development, and mutations in the TCOF1 gene are responsible for over 90% of TCS cases. The knowledge about the molecular mechanisms responsible for this syndrome is relatively scant, probably due to the difficulty of reproducing the pathology in experimental animals. Zebrafish is an emerging model for human disease studies, and we therefore assessed it as a model for studying TCS. We identified in silico the putative zebrafish TCOF1 ortholog and cloned the corresponding cDNA. The derived polypeptide shares the main structural domains found in mammals and amphibians. Tcof1 expression is restricted to the anterior-most regions of zebrafish developing embryos, similar to what happens in mouse embryos. Tcof1 loss-of-function resulted in fish showing phenotypes similar to those observed in TCS patients, and enabled a further characterization of the mechanisms underlying craniofacial malformation. Besides, we initiated the identification of potential molecular targets of treacle in zebrafish. We found that Tcof1 loss-of-function led to a decrease in the expression of cellular proliferation and craniofacial development. Together, results presented here strongly suggest that it is possible to achieve fish with TCS-like phenotype by knocking down the expression of the TCOF1 ortholog in zebrafish. This experimental condition may facilitate the study of the disease etiology during embryonic development.

A model of TCS was achieved by knocking-out the mouse Tcof gene. Homozygous are lethal and neonatal Tcof1+/− mice die within 24 h of birth [13]. Tcof1−/− mice show fewer mature ribosomes in neuroepithelial and neural crest (NC) cells [13,14], which were described as the expression territories of Tcof1 during embryonic development [13,15]. Recent results show that inhibition of p53 rescues craniofacial abnormalities by preventing apoptotic elimination of NC cells (NCC) [16]. Nevertheless, the ribosome biogenesis was not restored to wild-type levels in rescued mice [16]. This finding suggests that TCS phenotype is not only due to defects in ribosome biogenesis and, moreover, makes imperative to carry out further researches to fully elucidate the etiology of this pathology.

Introduction

Treacher Collins syndrome, also known as mandibulofacial dysostosis (TCS, OMIM #154500), is an autosomal dominant craniofacial malformation affecting 1:10,000 newborns. The affected structures have a common embryological origin in the first and second branchial arches. The resulting phenotype is characterized by downsloping palpebral fissures, lower lid coloboma, malar and mandibular hypoplasia, high or cleft palate, external ear malformations, atresia of the hearing canal, and conductive hearing loss [1]. There is striking clinical variability, with the most severe cases leading to perinatal death due to respiratory distress, and mild cases that escape clinical diagnosis. Despite this large spectrum of variability, penetrance is thought to be complete [2].

Mutations in the Treacher Collins-Franceschetti syndrome 1 gene (TCOF1, OMIM #606847), mapped to chromosome 5q32-q33.1, are responsible for over 90% of TCS cases. Over 120 pathogenic mutations have been identified in TCOF1. This observation suggests that haploinsufficiency of TCOF1’s protein product, treacle, is the underlying cause of TCS [3]. The majority of mutations in TCOF1 result in truncated treacle proteins [4–9], highlighting the importance of the C-terminal domain for treacle function. Treacle appears to participate in ribosome biogenesis by controlling pre-rRNA synthesis or processing [10–12]; however, the treacle biological role has not been fully understood yet.

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zebrafish and detected a dynamic spatiotemporal pattern similar to that observed for Tcof1 in mouse. The knockdown of B8JIY2/tof1 expression adversely affected the NC development and, furthermore, resulted in fish showing typical features of TCS patients. Remarkably, only craniofacial development was affected since the rest of embryonic structures developed normally in B8JIY2/tof1 knockdown zebrafish. Besides, we initiated the identification of potential molecular targets of treacle in zebrafish. Together, results strongly suggest that is possible to achieve fish with TCS-like phenotype by knocking down the expression of the TCOF1 orthologous in zebrafish. This experimental condition may facilitate the study of the disease etiology during embryonic development.

Results

In silico identification of B8JIY2 as the putative ortholog of TCOF1 in zebrafish

There was initially no information related to tof1 gene in zebrafish genomic databases. Therefore, we began our analysis using the tof1 cDNA sequence from Xenopus laevis (GenBank accession number AY731504) to search for homologous sequences in the Danio rerio cDNA_ALL Ensembl database (blastx, Assembly Zv9). Two alignments with a high percentage of identity were located on a region of the minus strand of chromosome 13 (Table S1 and Figure 1A; Chromosome13: 4,655,685–4,665,683). This region of chromosome 13 contains the Ensembl predicted genes B8JIY2_DANRE, QZUM1_DANRE, and nuclear and coiled-body phosphoprotein 1-like or nolc1l [19]. QZUM1_DANRE and nolc1l sequences partially overlap with the complete region covered by the B8JIY2 sequence (Figure 1A). On the contrary, B8JIY2_DANRE consists of 14 exons that span 19,250 bp in the zebrafish genome (Figure 1A). The predicted protein has 1,001 amino acid residues, a molecular weight of 102,031.33 Da, and an isoelectric point of 9.85. In a similar fashion than in treacle [20,21], the most relevant features of the B8JIY2-derived polypeptide are the presence of an amino terminal LsH dimerisation motif, three TCS treacle-like domains and three histone H5 motifs (Figure 1B). More than 100 putative Ser-phosphorylation sites were predicted using the NetPhos 2.0 application [22], most of which are located within the clusters of serine-acidic residues present within the repeat units (Figure 1C). Finally, because of the high homology observed between mammalian TCOF1 and Nolc1 we assessed the percentage of similarity among them and the zebrafish B8JIY2. Data revealed higher similarity of B8JIY2 with Tcof1 than with Nolc1 (Table S2). Collectively, these results strongly suggest that B8JIY2 is the TCOF1 ortholog in zebrafish. The B8JIY2 sequence was submitted to Gene Bank as the zebrafish tof1 gene under submission ID 1430387.

We designed primers to amplify a fragment specific to full-length zebrafish tof1 cDNA. RT-PCRs combining different sets of oligonucleotides led to the amplification of two transcripts, which were named B8JIY2a and B8JIY2b. In silico analyses revealed that B8JIY2b lacks 103 b of exon 11 (Figure S1). This could be due to either differential pre-mRNA processing or, alternatively, RT-PCR artifacts because the sequence lost in B8JIY2b corresponds to a genome repetitive region.

Tcof1 is expressed in a dynamic spatiotemporal pattern

The following challenge was to analyze the tof1 expression pattern. By whole-mount in situ hybridization (Figure 2) Tcof1 mRNA was detected ubiquitously in early staged embryos (Figure 2A–C), indicating a maternally inherited origin. Expression was still ubiquitous in 15 hours post-fertilization (hpf) embryos; however, the optic primordium and prospective brain regions (dotted oval in Figure 2D) showed more intense labeling. At 24 hpf, expression was mainly detected in the prospective eye, midbrain, hindbrain, and the NCC population that later forms a portion of the pre-mandibular, mandibular, first and second branchial arches (Figure 2E–J). At 40 hpf embryos, tof1 expression was detected in the retina, pectoral fin buds, pharyngeal arches, prospective craniofacial structures, and olfactory pits (Figure 2L–M). From 48 hpf onwards, the expression observed in brain was refined into a specific pattern along the anterior, dorsal, and posterior edges of the optic tectum (dotted lines in Figure 2L and P). At 72 hpf, tof1 expression was observed in the edges of the optic tectum, lenses, and craniofacial structures (arrowheads in Figure 2M, O–P, and dotted oval in R). Later on, 120 hpf-larvae showed expression in the craniofacial structures (arrowheads and dotted circles), lenses, and developing gut (Figure 2S–T). Controls were performed using a tof1 sense probe (Figure 2E–H, K, N and Q). Together, results revealed that tof1 expression is restricted to the anterior-most regions of zebrafish developing embryos, similar to what happens in mouse embryos [13,15]. Noteworthy, zebrafish tectal tof1 expression profile was highly similar to those of genes involved in cell proliferation, including proto-oncogenes and cell cycle regulatory genes [23–26]. This finding is in agreement with previous report showing a role for Tof1 in cell proliferation control [16].

Developmental tof1 mRNA distribution was also examined by semi-quantitative RT-PCR (Figure 3). Zebrafish elongation factor 1α (efla) mRNA was used as a control for RNA quality and for setting linear amplification conditions. Semi-quantitative RT-PCR assays confirmed that tof1 is maternally inherited and expressed at high levels during the first stages of zebrafish embryonic development. The highest tof1 mRNA expression was observed at the 1-cell stage. Over the course of development, tof1 mRNA expression decreased reaching a minimum at 12 hpf. From 12 hpf onwards, tof1 expression increased, reaching a plateau at 48 hpf.

Together, results presented here revealed that the tof1 spatiotemporal expression pattern is highly dynamic over the course of zebrafish embryonic development. Expression was restricted to regions and developmental stages wherein most of the critical morphogenetic events responsible for craniofacial structure formation take place, including the formation and blending of the branchial arches with the rest of the developing face. Notably, tof1 expression decreases once the facial complex has formed, and by 48 hpf onwards, expression becomes reduced near to background levels.

Tcof1 loss-of-function results in typical TCS phenotype

TCS patients show mutations in the TCOF1 gene that result in proteins lacking the C-terminal region [4–9]. The loss of the C-termimnus leads to mislocalized proteins and consequently to treacle loss-of-function [20,21]. Importantly, overexpression of C-terminal truncated forms of treacle did not affect the performance of cultured cells, suggesting that TCS results from treacle haploinsufficiency [8,12]. In view of this, we reasoned that the most informative approach to assess the role of tof1 during zebrafish embryonic development would be to decrease tof1-encoded protein levels by generating C-terminal truncated proteins. To achieve this, we designed two morpholino oligonucleotides (MO; [27]) that specifically modify tof1 pre-mRNA splicing during embryonic development. One of these MOs, in6ex7-MO, was directed to the junction between intron 6 and exon 7 while the other one, ex7:in7-MO, was directed to the junction between exon 7 and intron 7. Both MOs targeted splice donor or acceptor sites in tof1 exon 7, thereby preventing its inclusion during the pre-
mRNA splicing process. In silico analysis predicted that the loss of exon 7 results in a premature stop codon, which generates C-terminal truncated treacle forms. This strategy had been successfully used in our laboratory to study the role of other proteins during craniofacial development [28]. Different amounts of MO were injected in 1-cell staged embryos to achieve detectable phenotypes along with the lowest embryonic lethality. Noteworthy, the injection of in6:ex7-MO alone produced higher lethality than the injection of ex7:in7 MO (not shown). The best results were obtained by co-injecting 10.7 ng of each MO per embryo or 21.4 ng of ex7:in7 MO per embryo (Table 1). Lower amount of MO did not result in detectable phenotypes.

Figure 1. Localization of B8JIY2 in the zebrafish genome. A: B8JIY2 localizes to chromosome 13, spanning the bases 4,646,686 to 4,674,683. The studied contig is represented with a blue rectangle. Blast hits are labeled in red, showing filled boxes for exons and empty boxes for introns. Immediately below, B8JIY2, nolc1l, and Q7ZUM1 exons are represented with brown boxes and introns with brown broken lines. Near the bottom, the CG % is shown in grey. B: B8JIY2 in silico translated amino acid sequence with LisH dimerization (red), TCS treacle like (green), and histone H5 domains (blue) underlined. C: NetPhos 2.0 predicted phosphorylation sites in B8JIY2 in silico translated amino acid sequence. doi:10.1371/journal.pone.0029574.g001
performed as described elsewhere [27]. Normal embryos without any phenotype after injection of both the MOs may reflect different genetic backgrounds among fish, similarly to what happens in humans.

Because MO injection may induce neural cell death by off-target activation of p53 and derivatives [29], we checked by real time quantitative RT-PCR (qRT-PCR) the transcriptional levels of p53, p21 and the N-terminal truncated p53 isoform (Δ113 p53).
mRNAs in MO-treated embryos at 24 hpf. While full-length p53 RNA levels were not significantly increased in any of the MO-injected zebrafish embryos, the expression of p21 was upregulated in MO-injected embryos and virtually absent in controls (Figure 4). In contrast, the expression of Δ113 p53 isoform, the most reliable marker to diagnostic MO off-target effects [29], was significantly upregulated in in6:ex7-MO- and ex7:in7-MO- injected embryos but did not changed in embryos co-injected with both MOs or in controls (Figure 4). These findings ruled out a MO off-target effect for the latter experimental condition and, therefore, the following experiments were carried out using embryos injected with both MOs or in controls (Figure 4). Why a combination of in6:ex7-MO and ex7:in7-MO does not cause MO off-targeting remains completely unknown.

Typical morphant phenotypes obtained by tcof1 loss-of-function are shown in Figure 5. In 15 hpf morphants, the distinctive optic primordium was malformed, the four prominent subdivisions of the brain, the telencephalon, diencephalon, midbrain and hindbrain, as well as the rhombomeres 2 to 6 were undetectable in a dorsolateral view (Figure 5A–B, black dotted line). At 20 hpf, the otic vesicle was barely detectable, and dark apoptotic zones started to appear in the retina and midbrain-hindbrain ventricles (Figure 5C–D, black dotted arch). Morphants at 25 hpf showed eyes reduced in size and opaque apoptotic zones in all brain structures (Figure 5E–F). The typical internal structures seen for the forebrain, midbrain and hindbrain regions in control embryos were severely misarranged in morphants. Besides, morphants showed the dorsal tip of the developing head reduced in extension, being less prominent than in controls (see red lines and arrowhead in Figure 5E–F). At 5 days post-fertilization (dpf), morphant larvae exhibited eyes reduced in size and a shortened and rounded rather than triangular head. Moreover, morphants showed frontonasal hypoplasia manifested by the absence of the typical protruding mouth in front of the eyes (Figure 5G–J). Larvae exhibited heart edema, gut malformations, and swim bladder not fully inflated (see labels in Figure 5J). It should be noted that during all the
indicated between parentheses for each gene. Significant differences are labeled with an asterisk and the p-values are

data from Table 1 and Figure 4. The x-axis shows different genes for each sample of MO-treated embryos and the y-axis shows gene expression levels relative to control embryos. Gene expression levels were normalized with ef1α and rpl14 expression. Significant differences are indicated with a bar and the p-values are indicated between parentheses for each gene.

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observed in the Tcof1−/− mouse and TCS patients, including hypoplasia of the jaw cartilages.

Identification of putative tcof1 target genes

By microarray and real time RT-PCR studies, several genes were found to be positively or negatively regulated by Tcof1 in the mouse NB N1E-115 cell line [32]. Among them, ndrg1 was up-regulated while thx2b and cnbp were down-regulated by Tcof1 knockdown [32]. Two genes Mapk14 and Ddx42 were found to be down-regulated with knockdown of Tcof1 but this was not further confirmed by quantitative real time RT-PCR [32]. In view of this, we wondered if the tcof1 loss-of-function affects the expression of such target genes in zebrafish developing embryos. The expression of putative tcof1 targets was assessed by qRT-PCR using total RNA purified from control and tcof1-depleted embryos staged at 24 hpf. Specific oligonucleotides were designed for each putative target as indicated in Material and Methods. In agreement with previous results [32], ndrg1 expression significantly increased, and thx2b, ddx42 and cnbp expression significantly decreased in tcof1-depleted embryos (Figure 8). In our experimental conditions, mapk14b expression was the only one that did not significantly changed [32].

Discussion

We report here the identification of the formerly called B8JIY2 sequence as the TCOF1 ortholog in Danio rerio. Tcof1 and Treacle primary sequences are poorly conserved among mammals and even less conserved among other non-mammalian species [10]. Indeed, human treacle has only 62% amino acid identity with the mouse counterpart protein, and only 19% identity with Xenopus treacle [33]. However, several factors strongly support our hypothesis that the cloned cDNA corresponds to the TCOF1 ortholog in zebrafish, including the LisH domain in the N-terminus; the histone H5 motifs in the C-terminus; its dynamic and anterior-most spatiotemporal expression pattern during embryonic development; its requirement for normal NC and craniofacial cartilage development; and its action on previously informed putative treacle targets. Based on these data, we propose that B8JIY2 is the zebrafish ortholog of the human TCOF1.

Cranial cartilages were adversely affected in zebrafish tcof1 morphants. These cartilages are derived either entirely or in part from NCC and underlying the craniofacial anomalies detected in zebrafish mutants. The number and organization of chondrocytes were abnormal, which may be a consequence of NC aberrant development. Tcof1 mutant mice show elevated levels of apoptosis within the neural plate, which may initially deplete the neural stem cell pool from which NCC are derived [13]. Therefore, increased apoptosis may eventually reduce the number of migrating cranial NCC, which ultimately results in cranial cartilage hypoplasia. In zebrafish, cranial NCC that originate from the anterior midbrain give rise to the medial ethmoid plate; cranial NCC at the midbrain–hindbrain boundary form the trabecular rods and lateral ethmoid plate; slightly more posterior cranial NCC are involved in the pterygoid process of the palatoquadrate; while even more posterior cranial NCC contribute to the Meckel’s cartilage and palatoquadrate [34–36]. In our tcof1-knockdown morphants, in situ hybridization using molecular markers showed pronounced reductions in the pre-migratory and migratory CNC expression domains, comparable to those in sox9b mutants (Yan et al, 2005). Therefore, the shorter trabeculae, reduced lateral and medial ethmoid plates and palatoquadrate, as well as the absence of pterygoid processes in tcof1 morphant are likely to result from a diminution of progenitors by apoptosis of a subset of migratory cranial NCC population.
Experiments carried out in mice have clearly showed that the craniofacial defects displayed by Tcof1+/− mutants are rescued by inhibiting p53 either genetically or pharmacologically [16]. However, the ribosome biogenesis was still surprisingly impaired in rescued mice. This finding suggests that treacle plays other functions during embryogenesis not related with ribosomal biogenesis or even non-nuclear. In this regard, our data from qRT-PCR opens a new spectrum of possible biological functions for treacle. We found that treacle depletion affects, directly or indirectly, the expression of ndrg1, tbx2, ddx42, and cnbp in developing zebrafish embryos. According to this, it seems that expression of tcof1 as well as those genes that are associated with tcof1 expression and its protein treacle is required for the proliferation of NCC.

The N-myc downstream-regulated gene 1 (Ndrg1) is a metastasis suppressor with anti-tumor function. It reduces metastasis of numerous tumors by up-regulating p21, via p53 independent-mechanisms [37]. Interestingly, a significant increment of p21 expression was observed in MO-treated embryos without affecting p53 expression levels (Figure 4). Furthermore, Ndrg1 inhibits cancer cell migration [37] and increases apoptosis in vitro and in vivo [38]. Hence, tcof1 knockdown may lead to up-regulation of Ndrg1 which in turn may induce apoptosis of NC precursors, likely by up-regulating the potent cyclin-dependent kinase inhibitor p21.

Transcription factors of the T-box family are required both for early cell-fate decisions, such as those necessary for formation of the basic vertebrate body plan, and for differentiation and organogenesis. In particular Tbx2b mediates cell migration during neural plate formation and neuronal specification in zebrafish [39]. Tbx2 is a known transcriptional repressor and specifically regulates p19ARF in mouse embryo fibroblasts [40]. Activation of p19ARF stabilizes p53 and leads to growth arrest. Therefore tbx2b down-regulation in tcof1 zebrafish morphants may lead to the suppression of p53 function and the concomitant induction of basic apoptotic events during NC formation.

Ddx42 is a putative ATP-dependent RNA helicase implicated in cellular growth and apoptosis. Ddx42 interacts with ASPP2, a major apoptosis inducer known to enhance p53 transactivation of proapoptotic genes. Over-expression of Ddx42 interferes with apoptosis induction by ASPP2, whereas Ddx42 knockdown

Figure 6. Zebrafish tcof1 morphants showed reduced NC-specifier gene expression patterns. Lateral (G, I, K, and M) and dorsal (A–F, H, J, L, and N) views of control (A, C, E, and G–J) and in6ex7-MO+ex7in7-MO-treated (B, D, F, K–N) embryos. Embryos at 14 hpf were analyzed for foxD3 (A–B and E–F) and sox9b (C–D and G–H) expression patterns using whole-mount in situ hybridization. Abbreviations: de, diencephalic; hb, hindbrain; mb, midbrain; mhb, mid-hindbrain; ov, otic vesicles; s, somites. Scale bar: 185 μm for A–N. doi:10.1371/journal.pone.0029574.g006
reduces the survival rate of cultured human cells [41]. Therefore, decreased levels of ddx42 might promote apoptosis of NCC by failure in ASPP2 controlling.

Finally, cnbp has been previously related to both cell proliferation control and craniofacial development. Indeed, cnbp codes for a multifunctional nucleic acid chaperone [42,43] involved in cell death and proliferation control [44], and its knockdown results in aberrant skeletogenic NCC development and severe defects in the pharyngeal skeleton [25,28,45]. Besides, cnbp developmental expression profile is highly similar to that observed for tof1 during zebrafish embryonic development [23,46]. Collectively, these findings lead us to speculate that loss of Cnbp function goes a long way towards explaining the tof1 morphant skeletogenic phenotypes. Therefore, the functional relationship between tof1 and cnbp and their role in craniofacial development will certainly be essential topics for further studies.

This work was designed to introduce the scientific community to the possibilities associated with studying TCS in zebrafish. Our principal findings include the establishment of TCS model in zebrafish, and the identification of a set of genes as putative candidates for explaining TCS symptoms and designing therapeutic prevention strategies for TCS. The studies we describe here are limited in scope, but it is our belief that further and more sophisticated use of this simple vertebrate species could lead to shed new light on potential therapeutic avenues for the prevention not only of TCS but also of other congenital craniofacial disorders which may share a similar etiology and pathogenesis.

Materials and Methods

Fish and embryo rearing

Adult zebrafish were maintained at 28°C on a 14-h light/10-h dark cycle as previously described (Westerfield et al 1995). All embryos were staged according to development in hpf or dpf at 28°C (Kimmel et al. 1995), as well as handled in compliance with relevant national and international guidelines. Protocols were approved by the Commission of Bioethics, Facultad de Cs. Bioquímicas y Farmacéuticas-UNR, which has been accepted by the Ministerio de Salud de la Nación Argentina (http://www.saludinvestiga.org.ar/comites.asp?num_prov=13).

RNA extraction and RT-PCR analysis

Total RNA from embryos at different developmental stages was obtained using TRIZOL® Reagent (Invitrogen) following the manufacturer’s instructions. Purified RNA was incubated with RQ1 DNase (Promega) and used to perform RT-PCR.
RNA was retro-transcribed with SuperScript II enzyme (Invitrogen) and either poly-dT or sequence-specific oligonucleotides. For B8JIY2 cDNA cloning, combinations of seven forward primers and six reverse primers were employed. The sequences of the oligonucleotides were: Fwd1_ B8JIY2 (AGCCACGTCCAAAC-CAGTG), Fwd2_ B8JIY2 (TCAGACGGAAGACGGAAAAAG), Fwd3_ B8JIY2 (TCGAGAGAAGCAGAGACAGC), Fwd4_ B8JIY2 (CTCCGATGAGGAAGATGCTC), Fwd5_ B8JIY2 (GTTACTCGTTTCTCGTGGAAAACA), Fwd6_ B8JIY2 (CAGGACACACGGTTCCTA), Fwd7_ B8JIY2 (GGCGGAGGAATCTTCATCTT), Rvs1_ B8JIY2 (GGTGTTTTAGACGGCTCTCTTC), Rvs2_ B8JIY2 (CGAACATCGCTCTCCGCTTTC), Rvs3_ B8JIY2 (GGGTGTTTAGACGGCCTCTCTTC), Rvs4_ B8JIY2 (CTGAGACACGCGTGGTTT), Rvs5_ B8JIY2 (GACTGGAGCTTGAGCTTGTCAG). The isoforms generated by MO treatment were analyzed with a specific B8JIY2 exon 6 forward primer (CTCTAGAAGAAGCAGGACAGC) and an exon 8 reverse primer (GACTGGAGCTTGAGCTTGTCAG). Products were resolved in 1.2% (w/v) agarose gels stained with Gel Green, cloned into pGEM-T Easy Vector System (Promega), and their sequences obtained by University of Maine DNA Sequencing Facility.

Ef1α RT-PCR was performed as a control and for setting conditions [47], which were established at 24 amplification cycles. For qRT-PCR, first strand cDNA was reverse-transcribed from total RNA purified from 24 hpf embryo as described above and using oligo(dT) primer. Reactions were performed using four different RNA purifications and three independent experiments using an Eppendorf realplex2 and standard temperature protocol were performed. Primer sequences for ef1α, rpl14, p53, p21 and D113 p53 were used as described elsewhere [29]. The resting primer sequences were obtained using Primer3 program, as follows: Ndg1f, GACCAAAACACACTGCTAAAGAT; Ndg1r, GAATGGAAGTGAAAGGCAGCTCTG; Thx2hf, CTTTCATC-TGCTCAACAGACTGCT; Thx2hr, GTGTTAGGGTGACGGAAAAGTC; Mepk14hf, CTTCACTGATTTTGCTCTGACTAT; Mepk14hr, ATACGCTAGACCTTTCCCACCTCTCC; Ddx42f, AGCTTCGGAACATGATGAACCT; Ddx42r, CGACAAGTGGTTTGGTACTGAG. Expected PCR products span at least one intron (except the D113 p53 fragment), to ensure amplification solely from the cDNA and not from genomic DNA. Ef1α and rpl14 were used as endogenous control for normalization analysis. Acquired data were analyzed using qBase software version 2.2, and p-values were obtained from t-student analysis.

Sequences analysis
cDNAs were sequenced by the DNA Sequencing Facility of University of Maine. Complete mRNA and gene organization analysis were obtained from the www.ensembl.org website. Sequence alignments and similarity indexes calculations were carried out using ClustalW2 version 2.0.12 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The B8JIY2 sequence was submitted to Gene Bank as the zebrafish tcof1 gene under submission ID 1430387.

mRNA in situ hybridization
Embryos were staged and fixed overnight in 4% (w/v) paraformaldehyde (PFA) in 1× PBS at 4°C. After washing, embryos were stored in methanol at −20°C until use. Whole-mount in situ hybridization was carried out as previously described [48]. Digoxigenin-UTP-labeled sense and anti-sense riboprobes were prepared according to the manufacturer’s instructions (Roche Diagnostics, Mannheim). B8JIY2 exon 11 was used as
the detection probe. FoxD3 and sox9b expression patterns were analyzed as described elsewhere [25].

Antisense morpholino oligonucleotides

Embryos were obtained by natural mating and injected at the one-cell stage into the yolk immediately below the blastomeres using a gas-driven microinjection apparatus (MPFF-2 Pressure Injector, Applied scientific Instrumentation; Eugene, OR, USA). MOs were designed and synthesized by Gene Tools (Philomath, OR). The ex6:ex7-MO sequence is: 5'-AGCTTGATGAAGAGGTGCGAATAT-3' and the ex7:in7-MO sequence is: 5'-ATGAGAAAAGTGCAATACCAGTC-3'. Embryos were injected with 5 nl of the optimum concentration of MO prepared in 1× Danieu [27].

Alcian Blue staining

Four dpf larvae were fixed in 4% (w/v) PFA in 1× PBT, washed in 1× PBT four times, and treated as described elsewhere [49].

Supporting Information

Figure S1 Multiple sequence alignment of B8JIY2a, B8JIY2b, and B8JIY2 using Clustal 2.0.12. B8JIY2a and B8JIY2b sequences correspond to the two amplified, cloned and sequenced versions while B8JIY2 was obtained from Ensembl database. The alignment shows the deleted region presented in B8JIY2b sequence at around 1548 bases. (TIF)

Figure S2 Sequence alignment of tcof1 and tcof1ex7 using Clustal 2.0.12. The alignment highlights that the protein translated from the tcof1exon7-deleted version, which is generated in embryos injected with specific Morpholinos, do not have the three Histone H5 C-terminal motifs. (TIF)

Table S1 Results from the cDNA_ALL database of Ensembl (tblastx) search using the tcof1 Xenopus laevis cDNA sequence as query. (DOC)

Table S2 Similarity indexes for the alignment of Tcof1 and Nolc1 sequences from H. sapiens, R. norvegicus, M. musculus and X. laevis, and B8JIY2 and Q7ZUM1 from D. rerio. Sequence alignments and similarity indexes calculations were carried out using the software package GeneDoc ver. 2.6. (www.psc.edu/biomed/genedoc). (DOC)

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Author Contributions

Conceived and designed the experiments: AMJW NBC. Performed the experiments: AMJW NLS. Analyzed the data: NBC AMJW. Contributed reagents/materials/analysis tools: NBC. Wrote the paper: AMJW NBC.

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