The Mych Gene Is Required for Neural Crest Survival during Zebrafish Development

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Abstract

Background: Among Myc family genes, c-Myc is known to have a role in neural crest specification in Xenopus and in craniofacial development in the mouse. There is no information on the function of other Myc genes in neural crest development, or about any developmental role of zebrafish Myc genes.

Principal Findings: We isolated the zebrafish mych (myc homologue) gene. Knockdown of mych leads to severe defects in craniofacial development and in certain other tissues including the eye. These phenotypes appear to be caused by cell death in the neural crest and in the eye field in the anterior brain.

Significance: Mych is a novel factor required for neural crest cell survival in zebrafish.

Citation: Hong S-K, Tsang M, Dawid IB (2008) The Mych Gene Is Required for Neural Crest Survival during Zebrafish Development. PLoS ONE 3(4): e2029. doi:10.1371/journal.pone.0002029

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Introduction

Myc genes function in cellular proliferation by regulating cell cycle progression, apoptosis, and cell transformation. Myc factors are thought of as regulators of gene transcription that activate or repress multiple target genes [1,2,3,4,5]. Myc proteins have two recognized functional domains, the N-terminal domain (NTD) and the C-terminal domain (CTD). The CTD contains a basic helix-loop-helix leucine zipper (bHLH-LZ) motif that is necessary for target DNA binding and regulation of gene expression. The Myc NTD contributes to the control of transcriptional activation or repression of downstream target genes [6,7,8,9]. While the role of the Myc family in cell proliferation and cancer has received wide attention, comparatively less is known about its developmental functions.

Neural crest cells originate at the edge of the neural plate and at the dorsal aspect of the neural tube, and migrate to many locations where they differentiate into a great variety of cell types [10]. In Xenopus, c-Myc is expressed in early premigratory neural crest cells, and inhibition of its expression results in a loss of neural crest precursor cells and their derivatives [11]. The function of c-Myc in the neural crest involves Id3, a gene likewise expressed in neural crest precursors [12]. In the mouse, conditional inactivation of c-myc using Wnt1-Cre for targeted inactivation, led to skull, middle ear, and coat pigmentation defects [13]. These findings implicate c-Myc in the regulation of neural crest formation in the mouse. In zebrafish, Myc genes have been cloned and their expression has been reported [14,15]. Here we present isolation of a novel member of the family, mych (for nomenclature see Zebrafish Information Network: http://zfin.org), and report its expression pattern and a functional analysis using morpholino-based depletion. Mych knockdown embryos experience excess apoptotic cell death in the neural crest population and in anterior brain. Subsequently, pharyngeal arches failed to develop properly and the eyes were smaller than normal and lacked laminar organization. Thus, mych function is required in neural crest and eye cell survival and development.

Results

Isolation of full-length mych cDNA and sequence analysis of Mych protein

A partial mych cDNA was identified previously in an in situ hybridization-based gene expression screen [16]. We cloned full-length mych containing 1,975 base pairs (bp) using the 5′-RACE method. The cDNA predicts a 360-amino acid protein that contains a bHLH-LZ domain with high sequence identity with the CTD of vertebrate N-myc and C-myc proteins (57%–72%) (Fig. 1D). However there is only 39–57% identity in the NTD of Mych as compared to other Myc family proteins, and the amino acid sequences of the entire proteins are only 36–38% identical. A phylogenetic tree of the Myc family based on the CTD shows that Mych is located between the c-Myc and N-Myc clusters, closer to N-Myc (Fig. 1C). The mych gene is located on chromosome 6 according to the Sanger genome center, version Zv7 (http://www.ensembl.org/Danio_rerio/index.html; Zv7 Scaffold638, contig BX649289.11), and confirmed by radiation hybrid mapping using the LN54 panel [17] (Fig. 1A). To address the intracellular localization of Mych protein, a Flag-tagged mych construct was transfected into NIH3T3 cells; the protein localized in the nucleus, as is generally true for Myc family proteins (Fig. 1B).
Dynamic and specific expression of *mych* during early development

The expression of *mych* in developing zebrafish embryos was examined by whole mount in situ hybridization. Maternal transcripts are broadly expressed and appear to persist at least through blastula stages (Fig. 2A). After shield formation, *mych* expression is excluded from the dorsal marginal area but is retained in the future prechordal plate region (Fig. 2B). During gastrulation, *mych* transcripts are found in distinct dorsal and ventral expression domains in the embryo (Fig. 2C,C'). To interpret the early brain expression pattern of *mych*, we carried out double staining with eye, tectum, and prechordal plate markers, using *mab21l2*, *dnbx1a/mbx*, and *cstb1b/hgg1* [18,19,20]. At the 3-somite stage, *mych* is expressed in the eye field (Fig. 2E,E',F,F'), midbrain (Fig. 2F,F'), and prechordal plate (Fig. 2G,G'). The bilateral expression of *mych* in the hindbrain was analyzed by double staining with *eg2b/krx20*, a marker for rhombomeres 3 and 5. *Mych* is expressed in rhombomere 4 (Fig. 2F), and this expression is strongly increased in the neurogenic mutant *mib* [21], suggesting that *mych* transcripts are present in neuronal cells in rhombomere 4 (Fig. 2H). *Mych* expression in the presumptive hindbrain begins at 90% epiboly (data not shown), and thus the gene is a very early marker for this region. During midsegmentation stages *mych* is mainly expressed in the eye, midbrain, and somites (Fig. 2J,K). At 24hpf, mandibular, hyoid, and branchial arch expression increases (Fig. 2L), and eye expression is detected primarily in the photoreceptor layer at 36hpf (Fig. 2M). At 72hpf, brain, heart, and gut show strong *mych* expression (Fig. 2N,O). To test whether *mych* is expressed during neural crest development we carried out two-color in situ hybridization with *dlx2a* and *mych* (blue), to see whether *mych* expression is co-localized with *dlx2a* (red; Fig. 2P,Q), and at 32 hpf co-localization with *dlx2a* was seen in the pharyngeal arches (Fig. 2R,S). These observations indicate a wide but differential expression pattern for *mych* during embryonic development in the zebrafish.

**Mych** knockdown phenotypes

To study the function of *mych* in embryonic development, we used two morpholino oligonucleotides targeted against the 5'-untranslated region of the mRNA (UTR MO) and against the intron 1 splice donor site (SP MO). To determine MOs specificity, UTR MO was co-injected with mRNA for a fusion protein of Mych and GFP, showing a loss of GFP expression (Figure S1A,B). The SP MO specificity is examined by RT-PCR, using two sets of primers flanking the intron; the results show effective suppression of splicing (Figure S1C,D,E). *Mych* UTR MO injected embryos showed widening of the future trunk region at the 3-somite stage (75%, *n* = 109) (Fig. 3B and Fig. 4L,N) compared to control MO-injected embryos (5%, *n* = 77) (Fig. 3A and Fig. 4K,M). At 24hpf, UTR MO and SP MO-injected embryos (80%, *n* = 72) had a
small head and reduced trunk (Fig. 3C–F). To confirm that the two MOs affect the same process we injected a combination of half-maximal doses of each MO into the embryo and observed a similar phenotype (Fig. 3L) (85%, n = 88). The phenotypes elicited by each MO and by the combination of MOs were rescued by coinjection of mych mRNA coinjection (Fig. 3I: 73% rescued, n = 65; Fig. 3M,N: 85%, n = 94; Fig. 3O: 70%, n = 68). Head and eye defects were also seen at later stages (Fig. 3G–H,J–K), and layering of the retina failed to develop normally in the UTR MO-injected embryos at 48hpf (Fig. 3J-9, K-9).

Suppression of mych affects expression of anterior brain markers

To further analyze the mych UTR MO phenotype we carried out in situ hybridization using early brain markers. During gastrulation the expression of haxx1/ang, six3a, otx2, and zic1/opl were dramatically reduced in intensity and in the size of their expression domains (Fig. 5A–H). The regions marked by these genes contribute to the specification of the eye territory and the telencephalon [24]. By comparison, the reduction in the expression of hoxb1b in the posterior hindbrain and eve1 in the future trunk-tail were only slightly reduced in mych MO-injected embryos (Fig. 5I–L). Reduced anterior development in mych MO-injected embryos was also seen at the 3-somite stage. Mab21l2 and dmbx1a, marking the eye and midbrain anlagen, were reduced in size and expression level in mych MO-injected embryos (Fig. 5A–B), while the eye-specific gene rx3 showed a size but not intensity reduction (Fig. 4G,H). In contrast, the diencephalon marker barhl2 was not altered by mych MO injection (Fig. 4E,F).

In addition to genes that mark the anterior neural plate we also tested posterior brain and trunk markers after injection of mych MO. Double staining with pax2a and egr2b in the mid- and hindbrain indicates that these areas were not greatly affected by mych MO, although egr2b expression in rhombomere 5 is reduced.
Early neural crest specification requires Mych function

Mych is expressed in the mid- and hindbrain at early neural plate stages including the preplacodal regions (Fig. 2), and specifically in the early neural crest as seen by overlapping staining with the crest marker foxd3 (Fig. 2P,Q). This pattern suggests a possible function in neural crest specification which proceeds at this stage at the junction between the neural and non-neural ectoderm [25]. We tested the effect of injection of mych MOs on the expression of early neural crest marker foxd3 (Fig. 6A–F). The foxd3 expression is dramatically reduced by mych UTR MO (Fig. 6B), SP MO (Fig. 6C), and combination of half-maximal dose of both MOs (Fig. 6E). We also tested several early neural crest markers such as snail1a, sox9b, and sox10 found them dramatically reduced in the midbrain region and completely lost in the hindbrain and trunk neural crest regions (Fig. 6G–I). To visualize the cellular context of the reduction in the expression of these genes we sectioned foxd3-stained embryos (Fig. 6M,N). The foxd3 positive cells in the mych MO embryo contain a reduced number of neural crest cells and a reduced thickness of the region at the neural plate-to-epidermis boundary (Fig. 6M′,N′). We suggest that the inhibition of Mych
expression led to a loss of neural crest precursors in the embryo, as supported by experiments below.

**Mych knockdown disrupts pharyngeal arch development**

At 24hpf, *mych* is strongly expressed in the developing pharyngeal arches and maintained in this area for at least two days of subsequent development (Fig. 2). We observed effects of *mych* depletion on pharyngeal development by several approaches. Whole mount in situ hybridization at 26hpf showed that *dlx2a* and *tbx1* expression was lost in the branchial arches, while defects in the mandibular and hyoid arches were comparatively mild (Fig. 7A and B, and data not shown). At 36hpf, *hand2/dhand* expression in the branchial arches was likewise inhibited by *mych* MO (Fig. 7C,D). Mesodermal and endodermal development was likewise strongly inhibited after *mych* MO injection, as visualized by *tbx1* [26] at 43hpf (Fig. 7E,F). We also found a loss of *myod* expression in MO-injected embryos, indicating a loss of pharyngeal muscle (data not shown). To test whether these defects lead to loss of arch tissue, we injected *mych* MO into the *fli1-eGFP* transgenic line in which the cranial neural crest is visualized by GFP fluorescence [27]. A loss of branchial arches was seen, whereas portions of the mandibular and hyoid arch structures were maintained (Fig. 7G,H). These early pharyngeal defects lead to the loss of most parts of the cranial cartilages while most of the neurocranium was maintained, as seen by Alcian blue staining at day 5 (Fig. 7I–L). These data indicate that Mych is required for pharyngeal arch development in the zebrafish embryo.

**Loss of mych leads to increased cell death in the early neural plate**

Myc family genes have been implicated in the regulation of cell proliferation. Therefore we tested whether inhibition of *mych* expression decreases cell division in the embryo. Using Phospho-Histone H3 antibody to identify proliferating cells we found no substantial difference between control and *mych* MO-injected embryos (data not shown).

As the phenotypes resulting from *mych* depletion might also be caused by cell death, we tested for cell death at different times during gastrulation to segmentation stages. The earliest cell death was observed at about the 80% epiboly stage in the anterior dorsal region, with a moderate increase in the number of TUNEL-positive cells (data not shown). At bud stage, *mych* MO-injected embryos showed highly increased numbers of apoptotic cells, especially at the lateral edge of the neural plate and in the brain (65%, n = 67), as compared to control MO injected embryos, 95% of which showed very low levels of TUNEL-positive cells (n = 45) (Fig. 8A,B). The cell death phenotype was rescued by *mych* mRNA in 80% (n = 76) (Fig. 8C). In the neural plate of such embryos at the 3-somite stage, a dramatic increase in TUNEL-positive cells was observed in 85% (n = 45) of *mych* MO-injected embryos (Fig. 8E,F), as compared to 1% (n = 34) in control MO-injected embryos (Fig. 8D,D'). Again this phenotype was rescued by injection of *mych* mRNA in 80% (n = 63) of the embryos (Fig. 8E,F'). For statistical analysis, we counted the number of TUNEL-positive cells in the anterior neural plate in 20 embryos for each injection condition; the changes are highly significant (Fig. 6G). These data indicate that *mych* is important for the survival of neural plate cells in early embryo development.

**Discussion**

We have isolated the *mych* gene as a novel member of the Myc family in zebrafish, and have shown that it is involved in the survival of cells in the neural plate including the region from which the neural crest is derived. Zebrafish Myc family genes, including *c-myc*, *N-myc*, *L-myc* and *max* have been isolated previously and the distribution of their transcripts has been reported [14,15]. The Mych protein is related to other Myc proteins, showing low sequence similarity in the N-terminal Myc domain (NTD), but high similarity in the bHLH-LZ domain. As other Myc proteins, Mych is localized in the nucleus and may therefore function as a transcriptional factor (Fig. 1). Mych mRNA is expressed maternally, is restricted to a dorsal and a ventral domain at gastrulation, and later shows a dynamic expression.
pattern with a high level of expression in certain regions of the anterior brain. For comparison we carried out in situ hybridization with zebrafish c-myc and its binding partner max. Both were present maternally and were later expressed in a very broad pattern (data not shown). In contrast, myc expression was only zygotic, and its tissue distribution in the endoderm, retina, midbrain, hindbrain and branchial arches is similar to that of mych [15]. While the mych expression pattern at gastrulation suggests an involvement of this gene in dorsal-ventral patterning we found no substantial changes in the expression of bmp2, chordin, and goosecoid in mych MO-injected embryos (data not shown).

Inhibition of mych expression resulted in reduced size of the anterior brain without major changes in patterning (Figs. 4 and 5). At later stages we observed strongly reduced size of the head and eyes. These phenotypes may be due to the fact that mych knockdown greatly increases the level of cell death in the anterior neural plate (Fig. 8). While several tissues are affected in mych MO-injected embryos, neural crest differentiation seemed especially sensitive to the loss of Mych function. Early markers of neural crest differentiation were strongly reduced by mych knockdown (Fig. 6), and the development of branchial arches was severely disrupted, while the effect was less extensive in the mandibular and hyoid arches (Fig. 7). Neural crest cells arise at the border of neural and non-neural domains, and subsequently migrate to multiple target organs [10]. After migration into the pharyngeal arches, neural crest cells undergo condensation and chondrogenic differentiation to form the cartilage elements of the developing craniofacial skeleton. Expression of mych is maintained in these regions during their differentiation. A function for mych is supported by the observation that knockdown of its expression causes reduction of known regulatory genes in the branchial arches, such as wox2a, and ensuing morphological malformations (Fig. 7). These effects may be a consequence of the increased apoptosis in mych MO-injected embryos, as disruption of branchial arch development in conjunction with apoptosis as a result of loss of Ap2 transcription factor has been observed previously in zebrafish [28,29]. The specific involvement of Myc family genes in neural crest development has been studied in Xenopus and in the mouse. In Xenopus, c-Myc is involved in early neural crest specification [11], and conditional deletion of c-Myc in the mouse results in neural crest defects, including reduction of skull size and deficits in coat pigmentation and hearing [13]. In Xenopus, c-Myc appears to have a role in maintaining neural crest stem cells by acting through its target Id3 to prevent premature differentiation [11,12]. Zebrafish often contains additional members of gene families as compared to tetrapods, due to a genome duplication during evolution [30]. The resulting paralogs often have partly overlapping, partly distinct functions that together correspond to the functions of the single ortholog in other vertebrates. Therefore it is possible that in zebrafish mych takes on some of the functions carried out by N-Myc and c-Myc in Xenopus or the mouse. Such a model might explain the role of mych in neural crest development, although it is not clear whether the molecular function of mych in zebrafish is similar to that of c-Myc in Xenopus. In spite of this uncertainty, the requirement for mych function in zebrafish neural crest development supports the view that Myc family members are essential regulators of neural crest development in all vertebrates.

Materials and Methods

Fish strains

Wild type zebrafish strains AB* were maintained according to The zebrafish book: A Guide for the Laboratory Use of Zebrafish (Danio rerio) [31]. The nmycl52b mutant line was obtained from Ajay Chitnis, and homozygote fli1-eGFP transgenic line from Brant M. Weinstein.

Isolation of full-length mych cDNA and RH mapping

The original partial clone 5144 [16] contains 1.6 kb, and was extended to 1.9 kb full-length mych cDNA using the SMART RACE cDNA Amplification Kit (Clontech). Radiation hybrid mapping was done with the LN54 panel [17], using two primer sets: (1) Forward 5’-GCCGCAAGAGGATCTGCGGACTT-3’, Reverse 5’-AGGCTAAACCTCCAGCTGGTTCAC-3’; and (2) Forward 5’-TCGGCCGCTTTCCGTCCTACCTTT-3’, Reverse: 5’-CAGTTGGAGAAAGTCTGTTGTCCTC-3’. Amino acid sequence comparisons and phylogenetic tree analysis were carried out with DNASIS MAX version 2.0 (MiraBio, Hitachi software).
RT-PCR analysis
To isolate to total RNA, 20 each of cont MO and mych SP MO injected embryos were collected and isolated using TRIzol reagent (Invitrogen). The first-strand cDNA synthesis was performed SuperScript III First-Strand System (Invitrogen). RT-PCR reactions accomplished using two following sets of primers: Forward F1-CTGGACCTGCAACAC CGGGCGGCTCGGCTTG/Reverse R1-GTGGAGCCGCGCACGCTTCATACTCA; Forward F2-GACAAGAGGCCGACAGATGGAATCACCAC/Reverse R2-GCAGCTGCTGCTGGCCGGCTGC7GTC7TC. The amplified genomic DNA were confirmed by sequencing analysis.

Whole mount in situ hybridization
Whole mount in situ hybridization and two-color in situ hybridization performed as previously described [32]. Both digoxigenin- and fluorescein-labeled antisense RNA probes were generated using an RNA labeling kit (Roche).

mych MO and rescue mRNA injection
Antisense oligonucleotide Morpholinos (MO) were designed and obtained from Gene Tools, LLC. The mych 5′untranslated MO (UTR MO) does not contain AUG translation start sites sequences. The sequence of the UTR MO is 5′-ACGTGGTAGTAACAGGAAAC-3′. The mych splicing MO was designed splicing donor regions of intron between exon 1 and exon 2 (Figure S1 C). The sequence of the SP MO is 5′-GCAAAAAGAGCTACCCAGAATCGCTAG-3′, the control MO is 5′-GGCTGCTGCTGCGCCGGCTGTCCTC-3′. In all experiments 10ng of Control MO, 4ng of mych UTR MO, and 5ng of SP MO were injected into one-cell stage embryos. Full-length mych mRNA was subcloned into pCS2+ or pCS2+-eGFP1 vectors and synthesized from MessengerMachine SP6 Kit (Ambion), and 30 pg mRNA per embryo were injected in rescue experiments.

TUNEL assay and immunocytochemistry
TUNEL assay was performed as described previously [32]. The localization of Mych protein was determined after transfection of flag-tagged mych DNA into NIH3T3 cells using FuGENE 6 transfection reagent (Roche). We used 1:1000 dilution of the mouse mono clonal anti-flag antibody (Sigma), and 1:2000 dilution of mouse Alexa 488 (Invitrogen) as a secondary antibody. DAPI was used to stain nuclei. The images were scanned in a Zeiss LSM 510 confocal microscope.

Alcian blue and Methyl Green staining
Pharyngeal cartilage staining was carried out as previously described [32] using Alcian blue (Sigma). The 0.5% methyl green (Sigma) solution was prepared in 0.1 M sodium acetate buffer (pH 4.2). After 5 minute staining, samples were rinsed in water and dehydrated in 95% ethanol.

Histology
For sections, dehydrated embryos were embedded in JB-4 plastic resin (Polyscience Inc.), and 7 μm sections were obtained using a Leica RM2165 microtome. The two-color double staining sample was embedded with 10% gelatin (Electron Microscopy Sciences) in 1XPBS and section was performed using a Vibratome 3000 (Ted Pella Inc.) with 10 μm thickness.

Supporting Information
Figure S1 Mych MO specificity. A-B, Mych:GFP signal detection at the bud stage after injection with [B] or without [A] mych UTR MO. C, Schematic drawing of mych SP MO design and two different sets of RT-PCR primers. D–E, RT-PCR shows that the SP MO eliminates the normal mature mRNA band. Embryos were collected at the 3-somite stage. Found at: doi:10.1371/journal.pone.0002029.s001 (8.19 MB TIF)

Acknowledgments
We thank Mark Rath for zebrafish maintenance, and Neil A. Hukriede for set up information of LN54 RH PCR and mapping methods.

Author Contributions
Conceived and designed the experiments: ID SH. Performed the experiments: ID SH MT. Contributed reagents/materials/analysis tools: MT. Wrote the paper: ID SH.

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