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Expression and regulation of the zinc finger transcription factor Churchill during zebrafish development

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Abstract

During gastrulation dynamic cell movements establish the germ layers and shape the body axis of the vertebrate embryo. The zinc finger protein Churchill (*chch*) has been proposed to be a key regulator of these movements. We examined the expression pattern of *chch* in zebrafish and studied the regulation of *chch* by FGF signaling. We observed zygotic expression of *chch* during early cleavage stages. Two lines of evidence demonstrate that *chch* is zygotically expressed prior to the mid-blastula transition. First, blocking transcription during early cleavage stages represses *chch* expression. Second, endogenous levels of *chch* transcripts increase between 1-cell and 16-cell embryos. *chch* remains widely expressed during blastula and gastrula stages but scattered cells express higher levels of *chch*. By somitogenesis, *chch* is expressed in the ventral-most cells of the embryo adjacent to the yolk. In addition, transcripts are also observed in superficial cells on the surface of the yolk, in presumptive mucous cells and keratinocytes. By 30 hpf transcripts are observed in anterior neural tissue and ventral cells adjacent to the yolk. Over the next three days *chch* expression is indistinct until 4 dpf when we observe expression in the pharynx and gut. We show that activation of FGF signaling during gastrulation is sufficient to induce *chch* expression. In addition, we demonstrate that blocking FGF signaling between the 4-cell and shield stages represses *chch* expression.

Results and Discussion

Churchill (*chch*) is a small, highly conserved zinc finger transcription factor identified in a differential screen to isolate genes induced by Hensen's node (Sheng et al., 2003). *chch* was proposed to function as a switch between the functions of FGF in neural and mesoderm induction (Sheng et al., 2003). Morpholino knockdown of *chch* in the chick epiblast results in inappropriate migration of cells through the primitive streak (Sheng et al., 2003). This demonstrates that *chch* represses the movement of epiblast cells and allows them to remain in the prospective neural plate where they subsequently give rise to neural tissue (Sheng et al., 2003). Overexpression of *chch* in *Xenopus* embryos results in suppression of the mesodermal marker *brachyury* (*Xbra*) in embryos and animal cap assays (Sheng et al., 2003; Snir et al., 2006). FGF4 or FGF8 are sufficient to induce *chch* expression in the chick. In *Xenopus*, *chch* expression is regulated by XLPOU91 which mediates FGF responsiveness (Snir et al., 2006).

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chch acts primarily via induction of Smad interacting protein 1 (Sip1) (Sheng et al., 2003; Snir et al., 2006). Sip1 is a direct regulator of *Xbra* (Verschueren et al., 1999) and E-Cadherin (Comijn et al., 2001) and binds activated forms Smad1/5 and Smad2/3 to repress their transcriptional activity (Postigo, 2003; Postigo et al., 2003). These functions may mediate the activity of *chch* as a regulator of cell movement and germ layer specification.

chch expression has only been described in the chick and in a cnidarian. In the chick, *chch* expression begins at stage 4 and is restricted to the prospective neural plate and the neural plate (Sheng et al., 2003). In the cnidarian, *Nematostella vectensis*, a *chch* homologue is expressed in the pharyngeal endoderm, in a structure that expresses several factors found in the Spemann organizer (Matus et al., 2007). *chch* expression has not been described in other species. We have characterized the pattern of expression of the zebrafish *chch* gene. Unlike in the chick, we observe a broad expression pattern that is not restricted to the neural plate. Furthermore, we find that *chch* is zygotically expressed prior to the mid-blastula transition. During blastula and gastrula stages *chch* expression is widespread but becomes restricted to ventral epidermis during somitogenesis. At 30 hpf *chch* is expressed in anterior neural tissue and ventral cells adjacent to the yolk. By 48hrs expression is weak and specific domains are not detected until 4 dpf, when *chch* transcripts are enriched in the pharynx and gut. Finally, we demonstrate that FGF signaling is required for *chch* expression but our data also suggest roles for additional factors in regulating *chch* expression.

1.1 Zebrafish *chch* is expressed prior to the MBT

We identified a zebrafish *chch* homologue by searching the zebrafish genome database (Sanger, www.ensembl.org/Danio_rerio) using the chick protein sequence. A single *chch* gene was identified (Genbank accession # BC085457). This sequence contains the entire coding sequence (339bp) as well as 5'- and 3'-UTR regions (82bp and 515bp respectively). The *chch* genomic structure contains 4 exons. The predicted amino acid sequence is 112 amino acids long and shares about 85% identity and about 70% similarity with the chick *chch* protein (as reported by Sheng et al. 2003).

The temporal and spatial expression of zebrafish *chch* was examined by RT-PCR and whole mount RNA *in situ* hybridization. *chch* transcripts were detected by RT-PCR throughout the first 48hrs of development (fig. 1). The *chch* expression pattern was examined by RNA *in situ* hybridization using two non-overlapping probes (one containing the 5'-UTR and the coding sequence, and a second containing only the 3'-UTR). Both probes gave similar results and only one is shown (fig. 2A–Q). During early cleavage stages, *chch* transcripts are detected throughout the cytoplasm of each cell, but also in a punctate spot in each cell. At some stages, 2 spots appear in many cells (fig. 2A–D). In the late 8-cell embryo, the 2 spots align with the cleavage planes of the cells. The punctate stain co-localizes with DAPI stain demonstrating that *chch* transcripts are within the nucleus (data not shown).

To determine whether the nuclear stain corresponds to new transcripts, zygotic transcription was blocked in wild-type embryos using Actinomycin D. Embryos were microinjected with Actinomycin D and *chch* expression was examined at the 64-cell stage by RNA *in situ* hybridization (fig. 3A–B). Following Actinomycin D treatment, the nuclear stain was lost. As a further test to confirm early zygotic transcription of *chch*, real-time PCR was performed on 1-cell, 8-cell and 16-cell embryos to determine if the relative amount of *chch* transcripts increases as development progresses. This analysis revealed an increase in *chch* expression over these stages (fig. 3C). Taken together, these data demonstrate that *chch* is transcribed prior to the mid-blastula transition and is likely also supplied as a maternal mRNA.

Early zygotic transcription of a gene has been thought to be rare, but examples have been characterized in *Xenopus* (Yang et al., 2002). Recently, a set of over one hundred zebrafish

genes that are transcribed prior to the mid-blastula transition were identified by microarray analysis (Mathavan et al., 2005), although *chch* was not identified in this screen.

At sphere and shield stages, *chch* is widely expressed but some cells express higher levels of *chch* (fig. 2E–G). Expression was observed in forerunner cells (fig. 2F) but a clear pattern of specific regions of the embryo with high *chch* expression could not be discerned. During somitogenesis, *chch* is weakly expressed throughout the embryo, but robust expression in the ventral-most cells of the embryo adjacent to the yolk is observed (fig 2 J–M'). *chch* is also expressed on the surface of the yolk, in presumptive mucous cells and keratinocytes (fig. 2K). *chch* expression was not detected in these cell types in other regions of the embryo. By 30 hpf *chch* transcripts are observed in anterior neural tissue and ventral cells adjacent to the yolk (fig 2M, M'). Expression remains weak and indistinct until 96 hpf when expression is observed in the pharynx, ethmoid plate and gut (Fig 2O–Q).

In the chick, *chch* expression was shown in the prospective neural plate starting at stage 4 and remaining until stage 13 (Sheng et al., 2003). This is in contrast to what we have shown, where expression of zebrafish *chch* is not limited to the neural plate, but rather is expressed throughout early development. The expression domain that we observe in the zebrafish pharynx may be homologous to the expression of *chch* in the pharyngeal endoderm in *Nematostella* (Matus et al., 2007).

1.2 FGF signaling is required for *chch* expression

Activation of FGF signaling was shown to be sufficient to induce *chch* expression in the chick (Sheng et al., 2003). However, that study did not determine whether FGF was required for *chch* expression. To determine if *chch* is regulated by FGF signaling in zebrafish, we examined *chch* expression following inhibition or activation of FGF signaling. FGF signaling was inhibited using a pharmacological inhibitor of FGF, SU5402 (Mohammadi et al., 1996). Embryos were placed into 60μM SU5402 for 2 hours at a series of time points between the 4-cell and shield stages. Treated embryos were washed in embryo medium and allowed to develop until late gastrulation, when *chch* expression was assayed by quantitative real-time PCR (fig. 4A). Inhibiting FGF signaling during cleavage, blastula and gastrula stages, results in about a 40% decrease in *chch* gene expression by late gastrulation.

To determine whether *chch* expression recovered between the stage when SU5402 was washed out and expression was assayed, we performed a similar experiment but examined *chch* expression at shield stage (6 hpf) (fig. 4B). These treatments resulted in about a 40% decrease in *chch* transcript levels by shield stage suggesting that recovery of *chch* expression does not occur.

To determine whether activation of FGF signaling is sufficient to induce *chch* expression and to address the time window for this response, FGF signaling was activated using an inducible FGF type I receptor (iFGFR-1) (Pownall et al., 2003; Welm et al., 2002). This construct becomes active with the addition of a synthetic molecule, AP20187, which results in FGF receptor dimerization and activation. Wild-type embryos were injected with iFGFR-1 mRNA and the effect of FGF activation on *chch* expression was examined by real-time PCR during late gastrulation (fig. 4C). *chch* transcript levels were not altered when FGF signaling was activated prior to shield stage. When FGF signaling was activated after shield stage, there was a 25% increase in *chch* mRNA levels and nearly a 50% increase when FGF was activated at 70% epiboly (mid-gastrulation). Since the half-life of *chch* transcripts is not known and message could be rapidly degraded, we also assayed *chch* induction at shield stage after blastula stage induction of FGF signaling with iFGFR-1. We did not observe induction of *chch* mRNA at shield stage (data not shown) suggesting that the failure to observe induction of *chch* mRNA following early activation of FGF signaling is not due to rapid turnover of message.

Together, these results demonstrate that zebrafish *chch* is regulated by FGF signaling. Since inhibition of FGF activity at various developmental stages has similar effects on *chch* expression, continuous FGF signaling is may be required for maintenance of *chch* expression rather than initiation of expression. The *chch* transcripts we observe may have been generated prior to application of the FGF inhibitor or may stem from FGF independent regulation of *chch* expression. One set of candidates that may also regulate *chch* is members of the Oct3/4 class of pou domain proteins. Recently, XLPOU91 was shown to regulator of *chch* in *Xenopus* (Snir et al., 2006).

1.3 Conclusions

Taken together our results have shown that in zebrafish, *chch* is widely expressed prior to somitogenesis. While it is maternally expressed, it also begins to be zygotically transcribed prior to the mid-blastula transition. Unlike in the chick we do not detect specific expression of *chch* in the neural plate during gastrulation. However, we detect *chch* expression in the pharynx, which bares similarity to the expression of *chch* in the pharyngeal endoderm in *Nematostella*. Finally, we demonstrate that in zebrafish, wild-type *chch* expression requires FGF signaling but that activation of FGF signaling with iFGFR1 can induce *chch* expression during gastrula stages but not blastula stages.

2. Experimental procedures

2.1 Zebrafish stocks and embryo maintenance

Adult TL wild-type zebrafish were maintained at 28.5°C. Embryos were produced by natural matings of adult fish, collected and raised at 28.5°C in embryo medium until desired stage according to Kimmel et al. (Kimmel et al., 1995).

2.2 Cloning of Churchill

The Churchill coding sequence was cloned into the StuI site of the pCS2 plasmid. The primer sequences used for cloning were: F-ATGTGTACCGGTTGTGTCCA and R-CTAGAAGAGAGGTGCTGTTTG

2.3 Whole mount RNA in situ hybridization and photography

Embryos of the desired stage were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C then stored in 100% methanol at -20°C. RNA *in situ* hybridizations were done as previously described (Thisse et al., 1993). Constructs for *chch* in situ, probes containing the 5'UTR and the coding sequence or the 3'UTR alone were used to synthesize probes. After in situ hybridization, embryos were washed in benzyl benzoate/benzyl salicylate (40:1), and photographed using a Zeiss Axiocam mounted on a Zeiss Axioplan microscope. Whole mount stained embryos were dehydrated, embedded in JB-4 resin (Ted Pella) and sectioned at 8µm.

2.4 Pharmacological treatments

FGF signaling was pharmacologically inhibited by placing embryos of the appropriate stage into 60 µM SU5402 (Calbiochem, La Jolla, CA.) for 2hours. Upon removal from SU5402, embryos were washed in embryo media and allowed to develop until collected for real-time PCR. FGF signaling was activated with an inducible FGF receptor 1 (iFGFR-1) construct. Embryos were microinjected with 2.5pg iFGFR-1 mRNA and activated at the appropriate stage with the addition of 1.25 µM AP20187 (ARIAD Pharmaceuticals, www.airaid.com/regulationkits) resulting in constitutive activation of the receptor. Embryos were left in AP20187 for 15 minutes then washed 3 times in embryo media for 5 minutes and allowed to develop until the appropriate stage. Transcription was inhibited by microinjection

with 400pM Actinomycin D (Sigma-Aldrich) or DMSO as a control and collected at the desired stage for in situ hybridization.

2.5 Analysis of gene expression by real-time PCR

Embryos were collected at the appropriate stage and placed in TRIzol reagent (Invitrogen) for RNA extraction. cDNA was synthesized from .5 µg mRNA with the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR was performed and analyzed as previously described (Londin et al., 2005), primer sequences used for *chch* were: F- TGTGTCCAGAAGCAATATCC and R- TCCTCCTCATCTTCATTAC.

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Figure 1. RT-PCR analysis of *chch* expression

RT-PCR of mRNA from staged wild-type embryos with *chch* specific primers reveals that *chch* transcripts are present during the first 48hrs of development at all stages analyzed. β -actin was run as a loading control.

Figure 2. Whole mount RNA *in situ* hybridization analysis of *chch* expression in zebrafish

The *chch* expression pattern was examined in zebrafish by RNA *in situ* hybridization. *In situ* hybridization probes to the 5'-UTR and coding region or just the 3'-UTR were used; both gave similar results and only one is shown. During cleavage stages, *chch* transcripts are detected in the cytoplasm of all cells. Concentrated stain is also detected in the nucleus of each cell (A–D). At sphere and shield stages, *chch* is widely expressed and some cells express higher levels of transcript including a subset of forerunner cells (E–G). At 80% *chch* is ubiquitously expressed throughout the embryo but the punctate stain is less apparent (H). From the three-somite stage through the 22-somite stage *chch* transcripts are weakly detected throughout the embryo with highest levels in the ventral most regions of the embryo close to the yolk (I–L)

and in a punctate pattern on the surface of the yolk (focus on the surface of the yolk) (K). In panel K, the black arrowheads mark presumptive mucous cells and the white arrowhead is a presumptive keratinocyte. At 30 hpf *chch* transcripts are enriched in anterior neural tissue and ventral cells adjacent to the yolk (M) and (M'). The section in M' is at the level of the hindbrain. At 48 hpf *chch* expression is weak and indistinct (N). At 96 hpf *chch* transcripts are detected in the pharynx (black arrowhead), gut (white arrowhead) and ethmoid plate (asterisk) (O–Q, section in P'). A–E, G are animal pole views. F and O are dorsal views; H–N and P are lateral views and Q is a frontal view. Abbreviations used are: hindbrain (HB), head mesoderm (HM), eye (Ey) and tectum (TE).

Figure 3. *chch* is zygotically expressed prior to the mid-blastula transition

To determine if the early nuclear stain observed represents new zygotic transcripts, embryos were microinjected with the transcription inhibitor Actinomycin D (B) or DMSO (A). Embryos were collected at the 64-cell stage and examined for *chch* expression by RNA *in situ* hybridization. Following Actinomycin D treatment, the nuclear stain is lost. As a further test to confirm early zygotic transcription of *chch*, real-time PCR was performed on 1-cell through 16-cell embryos to determine if the relative amount of *chch* gene expression is increased as development progresses. This analysis revealed an increase in *chch* expression over these stages (C). Together these results show that *chch* is being zygotically transcribed prior to the mid-blastula transition.

Figure 4. FGF signaling regulates zebrafish *chch* expression

To determine if FGF signaling regulates *chch* gene expression, *chch* expression was assayed by real-time PCR following inhibition (A–B) or activation (C) of FGF signaling. Wild-type embryos were placed in were treated for 2 hours in SU5402 to block FGF signaling at the indicated stage (x-axis) and collected at 90% epiboly (A) or shield stage (B) for real-time PCR. FGF signaling was induced by the addition of AP20187 to iFGFR1 mRNA injected embryos. Embryos were placed in the AP20187 at the indicated stage (x-axis) for 15 minutes then washed and collected at 90% epiboly for real-time PCR (C). The fold change in *chch* transcript levels (y-axis) is graphed relative to untreated control embryos (which is set at 1).