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Original Article

Blastomeres and cells with mesendodermal fates of carp embryos express *cth1*, a member of the *TIS11* family of primary response genes

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ABSTRACT The carp *cth1* gene, related to the mammalian *TIS11* family of primary response genes, encodes a novel fish protein with two putative CCCH zinc fingers. This report describes the RNA expression of this gene during cleavage, blastula and gastrula stages of carp embryos.
***Cth1* mRNA is present in all cleavage stage blastomeres as a maternal message. After the late blastula stage, the maternal expression decreases, revealing a spot of higher expression at the margin of the blastoderm of the dome stage embryo. Further decrease of the maternal message reveals a ring of *cth1* expressing cells at the blastoderm margin from the stage of 40% epiboly onwards. By α-amanitin treatment we established that this local *cth1* expression is of zygotic origin. At the onset of gastrulation the cells of the ct*h1* ring involute, starting with those in the shield region, and at approximately 60% epiboly the ring is fully involuted and occupies the hypoblast layer. All *cth1* transcripts have disappeared at completion of epiboly. We discuss a possible role for the putative cth1 protein during cleavage and gastrulation.**

KEY WORDS: *cth1, fish development, zinc finger, endoderm, mesoderm*

Introduction

A great deal of attention is being paid to the molecular mechanisms that underlie the induction and differentiation of germ layers in teleost fish, especially zebrafish (*Danio rerio*). Gastrulation forms the epiblast and hypoblast, two layers of cells that give rise to respectively the ectoderm, and the mesoderm and endoderm (Kimmel *et al.*, 1995). Initially, the prediction of a cell's future could only be made just prior to gastrulation (Kimmel *et al.*, 1990) as cell mixing was thought to randomly rearrange the positions of cells in the blastula (Warga and Kimmel, 1990). Recently it became clear that in spite of this cell mixing, mesoderm and endoderm arise from only a subset of cells in the blastula (Kimmel *et al.*, 1990; Helde *et al.*, 1994), namely the cells at the margin of the blastoderm (hereafter referred to as 'marginal cells'). These marginal cells undergo but limited mixing and therefore keep their position near the margin of the blastoderm (Wilson *et al.*, 1993; Helde *et al.*, 1994) and then occupy the germ ring. Cells in the germ ring involute or ingress during gastrulation (reviewed by Solnica-Krezel *et al.*, 1995) and become the hypoblast layer. Though inducing signals are provided to the blastomeres before the onset of epiboly and gastrulation (Bozhkova *et al.*, 1994; Mizuno *et al.*, 1996; they are not determined until midgastrulation (Ho, 1992; Ho and Kimmel, 1993). How this signaling leads to specification is still unresolved. Analysis of gene expression patterns in the margin of the blastoderm, for example of circumferentially expressed genes like *no tail* (Schulte-Merker *et al.*, 1992), *lim1* (Toyama *et al.*, 1995) and *snail* (Hammerschmidt and Nusslein-Volhard, 1993; Thisse *et al.*, 1993,1995), provided some insight in the process. In this light we describe the expression of a novel fish gene that may be involved in the formation of the mesendoderm (mesoderm and endoderm), in carp (*Cyprinus carpio*) embryos. Carp is a teleost fish with identical development to zebrafish. The gene was previously isolated by subtractive hybridization (Stevens *et al.*, 1996b) and we now identify it as belonging to the *TIS11* family of primary response genes. *TIS11* family members, also reported as *TTP*, *Nup475*, *TIS11*, *cMG1*, *CTH*, *ERF*, are expressed in a wide variety of tissues and species (see references in discussion) and are induced after treatment with tumor promoters and growth factors, independent of protein synthesis (Herschman, 1991; Kaneda *et al.*, 1992; Bustin *et al.*, 1994). As the encoded proteins contain highly conserved putative zinc fingers, they are generally thought to regulate gene expression.

Here we report that the carp gene is expressed in all blastomeres at cleavage and blastula stages and is subsequently restricted to cells at the margin of the blastoderm and the germ ring. During

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gastrulation, the cells with transcripts involute and contribute to the hypoblast. We discuss the possible mode of action and propose a developmental role for this gene.

Results

Sequence analysis

We previously isolated the carp *cth1* clone (Fig. 1) by subtractive hybridization of oocyte cDNA with that of early segmentation stage embryos (Stevens *et al.*, 1996b). The clone has a length of 1438 nucleotides (nt) with an open reading frame extending from position 133 to the stopcodon at 810. It encodes a protein of 226 amino acids (aa). The 3' untranslated region (UTR) of 626 nt is complete, as indicated by the presence of a polyadenylation signal (AATAAA). Four possible signal sequences of unstable mRNAs (ATTTA) are recognized in the 3'UTR (Shaw and Kamen, 1986).

By Fasta and Blast searches we identified a 65 aa motif within the deduced carp protein, which is the characteristic feature of the group of mammalian and fruitfly *TIS11*, and yeast *CTH* genes. Carp cth1 is most closely related to the mouse and human TIS11d proteins (69% identity in motif). Members of the TIS11/CTH class share an unusual putative zinc finger motif (CCCH) in a consensus sequence **C**-x6-G-x-**C**-x-Y-x3-**C**-x-F-x-**H**, which is repeated and preceded by the conserved YKTEL sequence (Fig. 2). In addition, the spacing between the two repeats is conserved (Fig. 2). The carp motif owns all these features and therefore we identified the carp gene as the first fish *TIS11*-like gene. We followed the nomenclature suggested for the yeast genes (Thompson *et al.*, 1996) and named it *cth1* (cysteine-three-histidine).

Northern blot analysis

Carp *cth1* transcripts were present from the oocyte stage until the end of gastrulation (see below). To obtain further information concerning transcript sizes and the decrease of the expression level we analyzed the *cth1* signal in successive developmental stages on a northern blot (Fig. 3). *Cth1* RNA was abundantly present during the oocyte and cleavage stages. Around 5 h (oblong stage), the expression decreased to a lower level and persisted throughout gastrulation stages. At the start of somitic segmentation, the expression had disappeared. The *cth1* mRNA had not reappeared at 24 h of development (Fig. 3). In addition, the northern blot demonstrated a one-sized transcript for all developmental stages analyzed.

Carp cth1 RNA expression in situ

Carp embryonic development (Neudecker, 1976; Stroband *et al.*, 1992, 1995; Stevens *et al.*, 1996a) is similar to that of the closely related zebrafish, *Danio rerio* (Kimmel *et al.*, 1995). Through cleavage and blastula stages, maternal *cth1* transcripts were present in all blastomeres of the embryo (Fig. 4A), without any apparent localized pattern.

The first unevenness in the expression pattern arose just before doming when the intensity of expression in the blastoderm had decreased (Fig. 3). This reduction of overall expression revealed a spot at the margin of the blastoderm (Fig. 4B, and F). Concomitant with the optical reduction of the maternal signal in the blastoderm, caused by its thinning during doming and epiboly, a ring of *cth1* expressing cells, including the former spot, was distinguished at the margin (Fig. 4C and G). The expression in these cells was observed during completion of epiboly, while the signal in other blastoderm cells gradually diminished and disappeared before the end of gastrulation (Fig. 4A-E).

Gastrulation begins at 50% epiboly with the involution of cells at the margin of the blastoderm (Kimmel *et al.*, 1995). Cells that remain in the outer layer form the future epiblast, those in the inner layer form the hypoblast. At the onset of gastrulation, *cth1* cells in the ring at the margin involuted, starting in the spot on the dorsal side (Fig. 4D). At approximately 60-70% epiboly, the *cth1* expressing ring of cells had completely involuted and occupied the early hypoblast layer (Fig. 4E and H). The *cth1* signal in the hypoblast decreased as gastrulation proceeded and was undetectable at the end of epiboly.

A maternal origin for cth1 transcripts in marginal cells?

The localized pattern of *cth1* transcripts at the margin of the blastoderm during epiboly and gastrulation raised the question whether these transcripts were of maternal origin, or resulted from *de novo* transcription of the embryonic genome. Unstable mRNAs, containing ATTTA instability motifs as found in the carp mRNA, can be stabilized by mRNA binding proteins (Jacobs *et al.*, 1996), and thus the circumferential *cth1* expression could be the result of selective stabilization of maternal transcripts in marginal cells, while those in other cells are degraded. Alternatively, this pattern could be zygotic as it arose after genome activation occurs (around the 10th cleavage in carp: Stroband *et al.*, 1992). To solve this question, we blocked the embryonic transcription with α-amanitin, a drug that inhibits RNA polymerase II. If the *cth1* pattern is still observed after administration of this drug, the circumferential expression must be of maternal origin.

α-amanitin treatment

Alpha-amanitin was injected into early cleavage stage embryos (n=167). These embryos appeared normal in cleavage stages, but their development became aberrant when epiboly started in untreated embryos. Though not completely arrested in their development since cell divisions were still observed, α-amanitin embryos did not undergo epiboly or gastrulation. They maintained a late-blastula like shape until degenerating after 10 h of development.

First, we confirmed the inhibition by α-amanitin of embryonic genome activation by the absence of transcripts from a zygotically expressed gene, zebrafish *goosecoid*. *Goosecoid* was not detected in these embryos, contrary to its normal expression in control carp embryos (Fig. 4I), demonstrating that zygotic gene expression was indeed blocked. *Cth1* expression in α-amanitin treated embryos was examined when untreated embryos had reached dome (Fig. 4J), 30% epiboly (Fig. 4K) and approximately 60% epiboly stages (Fig. 4L). Only the ubiquitous signal that resulted from the maternal expression in cleavage stages was detected.

The localized expression in the spot and in a ring around the margin as seen in control embryos lacked in perturbed embryos, indicating a zygotic origin of this pattern in normal development.

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   1  GCGTCTTTCATCTGGAATAGAGCAGCGGAGTCAAGTGTTTGTCCGGAGTGCAGTCGGGTT
  61  TAGCGTCACTTTTTAAACGACAACCAACCAAGTTTTGTTAAGCTGCTGTTGAGTTTTGTA
 121  AAGCTGTCTAAAATGTTTGAGACTAGTACAGATAACCTGTTTCTGTTCCCCACTGAAGGC
                M  F  E  T  S  T  D  N  L  F  L  F  P  T  E  G
 181  CTGAATGAGGCTTTCTTCCCTGAAGAGGGTTTAGCCAGTGGGAGCTTGTCTCTTGCCAAG
      L  N  E  A  F  F  P  E  E  G  L  A  S  G  S  L  S  L  A  K
 241  GCCTTGCTTCCCCTGGTTGAGTCCCCATCACCCCCGATGACGCCCTGGCTCTGCTCCACC
      A  L  L  P  L  V  E  S  P  S  P  P  M  T  P  W  L  C  S  T
 301  CGCTATAAGACAGAACTGTGCAGCCGATATGCTGAGACAGGTACCTGCAAGTATGCCGAA
      R  Y  K  T  E  L  C  S  R  Y  A  E  T  G  T  C  K  Y  A  E
 361  CGCTGCCAGTTCGCCCATGGACTCCATGATCTCCACGTACCCTCCCGTCATCCCAAGTAC
      R  C  Q  F  A  H  G  L  H  D  L  H  V  P  S  R  H  P  K  Y
 421  AAAACCGAGCTGTGCCGTACCTACCACACTGCTGGCTACTGTGTCTATGGCACACGGTGT
      K  T  E  L  C  R  T  Y  H  T  A  G  Y  C  V  Y  G  T  R  C
 481  CTCTTTGTGCACAACCTTAAAGAGCACAGGCCTGTCCGTCAACGGTGCAGGAACGTGCCT
      L  F  V  H  N  L  K  E  H  R  P  V  R  Q  R  C  R  N  V  P
 541  TGCCGTACCTTTCGTGCATTTGGGGTTTGCCCTTTGGTACCAGATGCCACTTCCTGCATG
      C  R  T  F  R  A  F  G  V  C  P  L  V  P  D  A  T  S  C  M
 601  TGGAGGGTGGTTCAGAATCAGATGGTGGAGAGGAAGAGCAAACCTGTCAACCTATGTCAC
      W  R  V  V  Q  N  Q  M  V  E  R  K  S  K  P  V  N  L  C  H
 661  AGTCCCAAGAGTGGAAGCCTCGAGGTGCCCTCTGTCGCACCTTCAGCGCTTTTGGTTTCT
      S  P  K  S  G  S  L  E  V  P  S  V  A  P  S  A  L  L  V  S
 721  GTCTCTATGGCACCCGTTGTCGATTCCAACATGGGCTTCCCAACTCGATCAAAGGTGTCA
      V  S  M  A  P  V  V  D  S  N  M  G  F  P  T  R  S  K  V  S
 781  ACTCAACCCACACATCCTGGCCTCATCAGATGACCAATAGGGGATCTCTTTCACCTGTGT
      T  Q  P  T  H  P  G  L  I  R
 841  CAGATGCGTGCTCGTCACAATCTCCACCGTCCTCGTCCCCTCCGTCTGCGTTGGCTTCGC
 901  GGTGTACCCTGAGGGTTCTGGTCCAGTCACACCACCATCGGTAGAAGCAGTAGCCAACAA
 961  CGCTTTCACCTTCAGCAGCCAACATCTGAATGACCTTCTGCTCCCCCTGGCCCTTCGGCT
1021  CCAGCAGCTGGAGAATGTTACCAATGCTGGTCCTCAAGATGCTGTGGATAAGCCACTGTT
1081  GTTAAGTCTGTGGCAAGATGATCCAAGAAGCTAACTGCTGTGAGCTTTAACTGTGACTGT
1141  ATTGTGCCAAATTTATATTAACAGTTTTTATTTAGTTGTGTACAGTGTCTCTTTGCAACC
1201  TATTTTTAACTGCCTTTTTTAATTCAAGTCTGTCTTGTACTTCTGAGGCCACTTATTTTT
1261  GACTGACTGAATGAAGCCCCTTAAGTGTAAATTGAGACCACTTTACTGGTGTATTTATGA
1321  AGGATTTGACTATGTATGTTTCTAAATTACGGATTTTAAAACTTTAATGACTTAAATTAT
1381  TTTAAAAACATCAGCATCCTTTGCTCTCAATAAAACCTCCTAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide sequence and predicted protein product of carp *cth1*. *The instability conferring motifs are single underlined and the polyadenylation signal is double underlined. The putative zinc finger is printed in bold.*

Discussion

Carp cth1 belongs to the TIS11 gene family

The *TIS11* family of primary response genes is characterized by a repeated sequence motif that is supposed to encode a novel type of zinc finger (CCCH). Zinc-binding by the putative finger was recently proven (Worthington *et al.*, 1996), and the secondary structure of the zinc binding domain described. Another shared motif among the *TIS11* genes is the YKTEL pentapeptide. The putative carp protein has the characteristic features of TIS11 proteins and therefore we identified it as belonging to the *TIS11* gene family.

Members of this family were found in mouse and rat (Herschman, 1991), humans (Nie *et al.*, 1995), *Drosophila* (Ma *et al.*, 1994), cow (Lai *et al.*, 1995) and yeast (Thompson *et al.*, 1996). Except for truly homologous TIS11 proteins, such as human TIS11d/mouse TIS11d (Ino *et al.*, 1995), human ERF-1/mouse TIS11b/rat cMG1 (Barnard *et al.*, 1993), the similarity outside the zinc finger domains between the different TIS11 proteins is low. Though the zinc finger domain of carp cth1 is quite similar to that of mouse and human TIS11d (70%), an overall a comparison reveals only little similarity (35%), suggesting that TIS11d proteins are not true equivalents of the carp protein. It is likely that many more *TIS11* genes are still to be discovered and a true homolog of carp *cth1* may be found among them.

TIS11 expression during development

Little is known about the developmental expression of *TIS11* genes in different species. In carp embryos, *cth1* transcripts were found until the end of gastrulation. Maternally supplied transcripts were ubiquitously spread over the blastomeres during the cleavage period. After the late blastula stage, these transcripts were degraded and had disappeared before the end of gastrulation. *Drosophila TIS11* (*DTIS11*) is also expressed during early embryonic development (Ma *et al.*, 1994). *DTIS11* is abundantly present all over the embryo during the rapid nuclear divisions, comparable to the expression of carp *cth1* during cleavages, and starts to decline when cellularization starts.

A localized pattern of carp *cth1* expression became distinct during epiboly when the maternal expression disappeared. First seen as a spot at the margin of the blastoderm at the dome stage, *cth1* expression completely encircled the blastoderm margin during epiboly and involuted when gastrulation started. As in carp, fruitfly *DTIS11* mRNA remains present after the

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          carp cth1   RYKTELCSRY AETGTCKYAE RCQFAHGLHD LHVPSRHPKY KTELCRTYHT AGYCVYGTRC LFVHN
       mouse TIS11d   RYKTELCRPF EESGTCKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
       mouse TIS11b   RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
       mouse TIS11a   RYKTELCRTY SESGRCRYGA KCQFAHGLGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIHN
       human TIS11a   RYKTELCRTF SESGRCRYGA KCQFAHGLGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIHN
human TIS11b/ERF1   RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
         human ERF2   RYKTELCRPF EESGTCKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
       human TIS11d   RYKTELCRPF EESGTCKYGE KCQFAHGFHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
          cow Zfp36   RYKTELCRTF SESGRCRYGA KCQFAHGLGE LRQASRHPKY KTELCHKFYL QGRCPYGSRC HFIHN
          rat TIS11   RYKTELCRTY SESGRCRYGA KCQFAHGPGE LRQANRHPKY KTELCHKFYL QGRCPYGSRC HFIHN
  rat TIS11b/cMG1   RYKTELCRPF EENGACKYGD KCQFAHGIHE LRSLTRHPKY KTELCRTFHT IGFCPYGPRC HFIHN
  fruitfly DTIS11   RYKTELCRPF EEAGECKYGE KCQFAHGSHE LRNVHRHPKY KTEYCRTFHS VGFCPYGPRC HFVHN
       yeast CTH1   LYKTELCESF TIKGYCKYGN KCQFAHGLNE LKFKKKSNNY RTKPCINWSK LGYCPYGKRC CFKHG
       yeast CTH2   LYKTELCESF TLKGSCPYGS KCQFAHGLGE LKVKKSCKNF RTKPCVNWEK LGYCPYGRRC CFKHG

          consensus   -YKTELC---  ---G-C-Y--  -CQFAHGL--  L--------y ktelC-----  -G-C-YG-RC  -F-H-
                            *          *       *   *                    *          *     *     *
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Fig. 2. Comparison of the zinc finger regions of carp cth1 and other members of the TIS11 family. *The two zinc binding motifs (CCCH) are spaced by 18 amino acids. Zinc binding residues are indicated by asterisks. The CCCH motifs are preceded by a conserved YKTEL sequence, which is less conserved in the second motif. The TIS11 sequences are available under the following accession numbers: carp cth1 (Y10163); mouse TIS11d (P23949); mouse TIS11b (B39590); mouse TIS11a (P22893); human TIS11a (P26651); human TIS11b/ERF1 (Q07352); human ERF2 (S49147); human TIS11d (U07802); cow Zfp36 (L42319); rat TIS11 (JC1255); rat TIS11b/cMG1 (P17431); fruitfly DTIS11 (U13397); yeast CTH1 (P47976); yeast CTH2 (P47977). The mouse and human TIS11a are also known as TIS11, Zfp36, Nup475, or TTP.*

cleavage period (Ma *et al.*, 1994) but it is not clear if these transcripts are localized, like the carp transcripts are.

If classes of *TIS11* genes exist, they may have distinct developmental expression patterns and gene-specific modes of transcriptional regulation. Differential regulation has already been demonstrated *in vitro* for *TIS11* and *TIS11B* (Varnum *et al.*, 1991). It is interesting to know to what extent the expression patterns of those *TIS11* classes are comparable between species. Conservation of developmental patterns is suggestive of conserved roles during development.

Maternal versus zygotic transcription

The question whether the localized *cth1* pattern during epiboly arose as a result of zygotic transcription or consisted of remnant maternal RNA is an intriguing one. On the one hand, localized zygotic expression preceded by maternal expression is commonly seen in embryos (for example *goosecoid*: Stachel *et al.*, 1993; *snail1*: Thisse *et al.*, 1993) and could explain the pattern of *cth1* in post-cleavage carp embryos.

On the other hand, arguments exist that favor the maternal origin of this pattern. Firstly, maternal *cth1* transcripts were still abundantly present when the localized pattern became distinct, in contrast to the maternal *goosecoid* RNA that disappears before zygotic transcripts appear (Stachel *et al.*, 1993). Secondly, zygotic transcription could be expected to increase the abundance of *cth1* mRNA, but the northern blot showed a decline of the level of expression instead.

Moreover, carp embryos showed no other size of *cth1* transcript than the maternal one, in contrast to fruitflies that have an embryonic transcript of different size (Ma *et al.*, 1994). These arguments led us to consider a maternal origin for the localized mRNA. The model that we proposed was one of selective stabilization of maternal transcripts, and would provide an interesting mechanism for localization of expression. *TIS11* RNAs rapidly degrade and this instability is attributed to ATTTA sequences (Shaw and Kamen, 1986) which are also present in the 3'UTR of carp *cth1*. A half-life less than 30 min is reported for TTP mRNA (Lai *et al.*, 1990). Since the *cth1* levels remained high until the age of 5 h, we expected the maternal *cth1* RNA to be stabilized. After the cleavage stages, a stabilization of the RNA might occur only in the margin of the blastoderm, giving rise to a circle of expression while transcripts in other parts of the embryo are degraded.

However, no localized expression was found after treatment with α-amanitin thereby making the existence of a mechanism of selective stabilization improbable. We conclude that the localized pattern most likely consists of zygotic *cth1* transcripts.

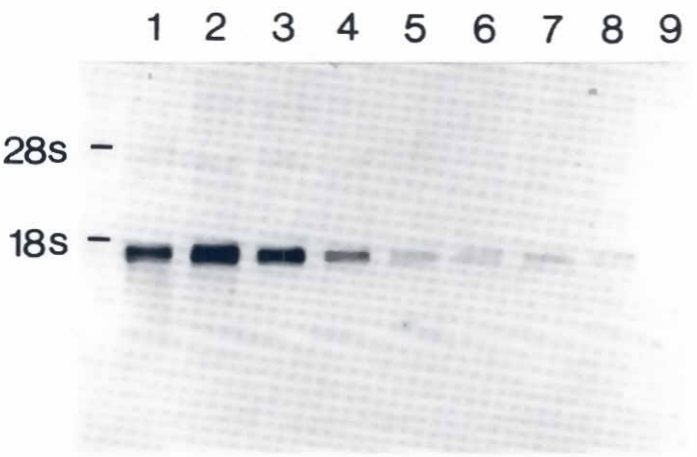

Fig. 3. Northern analysis of *cth1* RNA. *Lanes 1-9 contain 2 μg total carp RNA from: 1) 0 h (oocyte), 2) 2 h (32/64 cells), 3) 4 h (high), 4) 5 h (oblong), 5) 5 1/2 h (early dome), 6) 6 h (30% epiboly), 7) 6 1/2 h (40-50% epiboly), 8) 7 h (± 60% epiboly), 9) 24 h (late segmentation). Lanes 1 and 2 are respectively slightly underloaded and overloaded. The positions of the ribosomal RNAs are shown.*

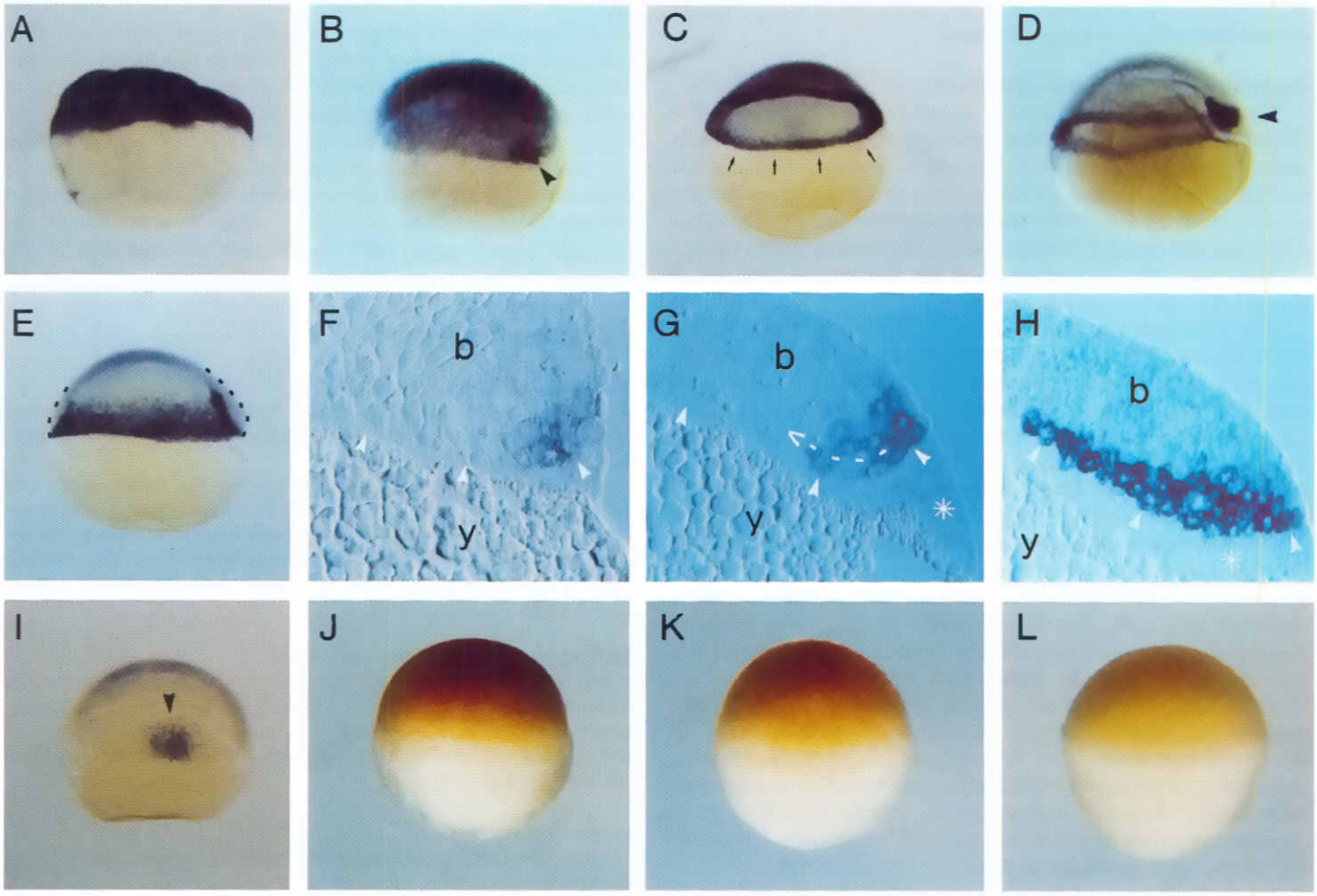

Fig. 4. *Cth1 RNA distribution* in carp embryos. (A) *Cleavage stage (16 cells) embryo has maternal cth1 RNA in all blastomeres.* **(B)** *Maternal cth1 expression has become less distinct. A spot of higher expression is visible at the margin of the blastoderm (arrowhead).* **(C)** *Forty percent epiboly; a ring of cth1 expressing cells is located at the margin of the blastoderm (arrows). Maternal RNA is still ubiquitously present at low level in the rest of the blastoderm.* **(D)** *Fifty percent epiboly; the cth1-ring involutes during gastrulation, starting in the shield region (arrowhead). Note that the half of the circle which faces away from the reader is visible through the yolk (upper line), whereas in (E) only the front half of this circle can be seen. The irregular shape of the cth1-circle is caused by unevenness of the blastoderm margin, which often occurs in carp epiboly.* **(E)** *The cth1-circle is fully involuted at 60% epiboly and occupies the hypoblast layer. The blastoderm outline is marked with a dotted line. Dorsal is to the right.* **(F-H)** *Nomarski images of sectioned embryos. Asterisks indicate the YSL, with arrowheads pointing at the border between blastoderm and YSL. y, yolk; b, blastoderm. (F) and (G) are understained, therefore the overall low-level expression in the blastoderm is not visible. (F) Section of B (dome stage), through the spot of cth1 expressing cells at the blastoderm margin.* **(G)** *Section through C (cth1-ring at 40% epiboly). Note that the blastoderm layer has thinned during epiboly (compare G to F). The cth1 expressing cells of the ring are localized in the margin of the blastoderm and will involute during gastrulation (arrow indicates direction of movement).* **(H)** *Section through E (60% epiboly) showing that cth1 expressing cells have involuted into the hypoblast. There still is overall low-level expression in the blastoderm.* **(I)** *Carp embryos (60% epiboly) show a normal pattern for zebrafish goosecoid (arrowhead).* **(J-L)** *Cth1 expression in -amanitin-treated carp embryos fixed when normal embryos had reached dome (J), 30% (K) and 60% epiboly (L). Cth1 RNA is stained brown instead of blue. The overall expression in the blastoderm is of maternal origin and disappears gradually, as in normal embryos (B,C,E). The localized expression pattern does not arise and is therefore most likely of zygotic origin.*

Induction of zygotic expression

Why is zygotic *cth1* transcription restricted to cells in the margin of the blastoderm? An explanation may be found in recent reviews (Grunwald, 1996; Stroband *et al.*, 1996) that suggest that maternally supplied substances, emitted from the yolk cell, are responsible for induction processes in marginal cells. The mammalian *TIS11* genes are rapidly induced by tumor promoters and growth factors (see references in introduction). The zygotic *Drosophila TIS11* RNA is also inducible (Ma *et al.*, 1994). By analogy it is likely that zygotic *cth1* expression in carp reflects a primary response to an inducing factor. The above model suggests that this substance, for example a growth factor (Stroband *et al.*, 1996), originates from the yolk cell. Cells in close contact with the YSL are thought to receive this inducing signal and this could

explain why *cth1* transcription is only activated in a ring of cells around the margin of the blastoderm.

Possible function of the cth1 protein

The RNA expression pattern suggests that *cth1* translation could happen twice; first from maternal mRNA in blastomeres during the cleavage and blastula periods, and then from zygotic mRNA in marginal cells during epiboly. However, many factors are known to regulate mRNA translation in embryos (Wormington, 1993; MacDonald and Smibert, 1996; Wolffe and Meric, 1996).

Therefore, *cth1* translation does not necessarily occur in all parts of the RNA expression pattern.

It is assumed that the mechanism by which TIS11 proteins act, involves the conceptual zinc finger. Zinc finger proteins are generally considered regulators of transcription (Pabo and Sauer, 1992). Recently, evidence for RNA binding by a *Xenopus* zinc finger protein (Andreazzoli *et al.*, 1993) suggested a second mode of action. Cytoplasmic Xfin protein is thought to bind target mRNAs and thereby regulate their translation. Phosphorylation of Xfin should lead to tight binding whereas dephosphorylation should release the target mRNA (Vignali *et al.*, 1994). Although, for the present, evidence for RNA-binding by TIS11/CTH proteins is lacking, three facts suggest a similar mode of action. Firstly, the existence of only transcriptional regulation would exclude a role for the maternal protein, as the fish genome is not yet transcribed during early cleavages. Thus, a role in translation is more plausible. Secondly, the mouse TTP protein is rapidly translocated from the nucleus to the cytoplasm in response to mitogens (Taylor *et al.*, 1996[b]), suggestive of a cytoplasmic function and thus favouring translational over transcriptional regulation. Thirdly, the TTP protein is phosphorylated and this may regulate its function (Taylor *et al.*, 1995), as suggested for Xfin.

As to the nature of gene regulation, inhibiting roles have been attributed to TIS11 proteins. For example, overexpression of CTH in yeast leads to impairment of growth and this is probably mediated through the zinc finger domain (Ma and Herschman, 1995; Thompson *et al.*, 1996). In mice, TTP is thought to have a role in negative regulation of TNFα (tumor necrosis factor) synthesis (Taylor *et al.*, 1996[a]). We propose that carp cth1 regulates gene expression by inhibition of translation of distinct target mRNAs. The impairment of the translation of mRNAs, coding for proteins that respond to inducing signals, could be a mechanism to time or prevent the selection of certain differentiation pathways. Such a function could be used in development to prevent premature restriction of cellular potential. In support of this hypothesis is the recent finding that a protein with TIS11-like zinc fingers (PIE-1) in *C. elegans* has a comparable mode of action and preserves pluripotency in germ cells (Mello *et al.*, 1996).

For both the maternal and the zygotic protein, a role in maintaining cellular potential is conceivable. During cleavages, blastomeres remain pluripotent (Kimmel and Warga, 1987; Helde *et al.*, 1994). Maternal cth1 protein could be involved in this. The zygotic cth1 may be implicated in the commitment of the mesendodermal fate. Expressing *cth1* RNA, the marginal cells involute during gastrulation and form the hypoblast layer, which gives rise to mesoderm and endoderm (Kimmel *et al.*, 1990; Warga and Kimmel, 1990). Though marginal cells receive mesoderm-inducing signals already early (Bozhkova *et al.*, 1994; Mizuno *et al.*, 1996), a hypoblast-fate is not determined until midgastrulation (Ho and Kimmel, 1993) and cth1 protein could perform a delaying role in this determination. Alternatively, the protein could bring about the choice for a particular fate. How cells acquire the endodermal fate is still mysterious. Perhaps it is the default choice of hypoblast cells that do not possess appropriate proteins to respond to mesoderm-inducing signals. It is tempting to speculate that by preventing the translation of such proteins in a subset of the marginal blastomeres, cth1 could favor the selection of the endodermal fate.

Materials and Methods

Embryos and α-amanitin injections

Carp oocytes were fertilized *in vitro*. Sperm and eggs were obtained by squeezing, after stimulation with pituitary suspension as described by Stroband *et al.* (1992).

Alpha-amanitin (Sigma-Aldrich Chemie BV, the Netherlands), was injected during the 4 to 32 cell stages, in a final concentration in the embryo of approximately 2-7 μg/ml (volume embryo was estimated 0.7 μl). For use *in situ* hybridization (ISH), embryos were fixed when controls (injected with equal volume 0.9% NaCl) had reached dome, 30% epiboly or 60% epiboly stages.

Cloning and sequencing of cth1 cDNA

Full length *cth1* was cloned from an oocyte stage cDNA library after subtractive hybridization screening (described in Stevens *et al.*, 1996b). Sequencing was performed as in that publication. The sequence described here is available from the EMBL database under accession number Y10163.

Northern blot analysis

Total RNA was prepared by the acid guanidinium thiocyanate-phenol-chloroform extraction method (Chomczynski and Sacchi, 1987). 2 μg total RNA per lane were electrophoresed through a 1.2% agarose/formaldehyde gel, transferred onto a Nytran membrane (Schleicher and Schuell Nederland BV, the Netherlands) and vacuum-baked for one hour at 80°C. Hybridization with Dig-labeled RNA probe (same as for ISH) was performed overnight at 68°C in 5xSSC/ 50% formamide/ 0.02% sodium dodecylsulphate/ 0.1% N-lauroylsarkosine/ 2% blocking reagent (Boehringer Mannheim (BM), Germany). Washes and chemiluminescent detection (CDP-star) were carried out as recommended by BM. Fuji X-ray films were exposed less than 1 min to visualize *cth1* transcripts.

Whole-mount In situ hybridization and cryo-sectioning

The ISH were carried out as in Stroband *et al.* (1995), with some modifications. Embryos were fixed in MEMFA (4% formaldehyde/0.1 M MOPS pH 7.4/2 mM EGTA/1 mM MgSO4), and washed in PBST (PBS buffer/0.1% Tween-20) before hybridizing. Methanol storage and proteinase K treatments were omitted. The *cth1* probe was diluted 2000 times in (pre)hybridization solution (50% formamide/ 2xSSC/2% blocking reagent/0.1% Tween-20/5 mg/ml yeast RNA/ 50 μg/ml heparin) and hybridized at 60-70°C. After staining at room temperature, embryos were stored in 4% paraformaldehyde in PBS at 4°C. For photography, embryos were usually cleared in benzyl benzoate: benzyl alcohol (2:1). The staining is brown instead of blue, in non-cleared embryos (Fig. 4J-L).

Sections of whole-mount stained embryos were prepared by cryo-sectioning. Stained embryos were fixed in 4% paraformaldehyde/ 0.2% glutaraldehyde/ PBS, rinsed 3 times in PBST (5 min each) and mounted in 1.5% agarose/5% sucrose. Following overnight immersion with 30% sucrose at 4°C, agar-mounted embryos were frozen in liquid nitrogen and then sectioned at -23°C.

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References

ANDREAZZOLI, M., DE LUCCHINI, S., COSTA, M. and BARSACCHI, G. (1993). RNA binding properties and evolutionary conservation of the *Xenopus* multifinger protein Xfin. *Nucleic Acids Res. 21:* 4218-4225.

BARNARD, R.C., PASCALL, J.C., BROWN, K.D., MCKAY, I.A., WILLIAMS, N.S. and BUSTIN, S.A. (1993). Coding sequence of *ERF-1*, the human homologue of *Tis11b/cMG1*, members of the *Tis11* family of early response genes. *Nucleic Acids Res. 21:* 3580-3580.

BOZHKOVA, V., TE KRONNIE, G. and TIMMERMANS, L.P.M. (1994). Mesoderm differentiation in explants of carp embryos. *Roux Arch. Dev. Biol. 204:* 20-29.

BUSTIN, S.A., NIE, X-F., BARNARD, R.C., KUMAR, V., PASCALL, J.C., BROWN, K.D., LEIGH, I.M., WILLIAMS, N.S. and MCKAY, I.A. (1994). Cloning and characterization of *ERF-1*, a human member of the *Tis11* family of early-response genes. *DNA Cell Biol. 13:* 449-459.

CHOMCZYNSKI, P. and SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem 162:* 156-159

GRUNWALD, D.J. (1996). A unifying model for the origin of the vertebrate organizer. *Neth. J. Zool. 46:* 22-46.

HAMMERSCHMIDT, M. and NÜSSLEIN-VOLHARD, C. (1993). The expression of a zebrafish gene homologous to *Drosophila snail* suggests a conserved function in invertebrate and vertebrate gastrulation. *Development 119:* 1107-1118.

HELDE, K.A., WILSON, E.T., CRETEKOS, C.J. and GRUNWALD, D.J. (1994). Contribution of early cells to the fate map of the zebrafish gastrula. *Science 265:* 517-520.

HERSCHMAN, H.R. (1991). Primary response genes induced by growth factors and tumor promoters. *Annu. Rev. Biochem. 60:* 281-319.

HO, R.K. (1992). Cell movements and cell fate during zebrafish gastrulation. *Development 1992 (Suppl.):* 65-73.

HO, R.K. and KIMMEL, C.B. (1993). Commitment of cell fate in the early zebrafish embryo. *Science 261:* 109-111.

INO, T., YASUI, H., HIRANO, M. and KUROSAWA, Y. (1995). Identification of a member of the *TIS11* early response gene family at the insertion point of a DNA fragment containing a gene for the T-cell receptor ß chain in an acute T-cell leukemia. *Oncogene 11:* 2705-2710.

JACOBS, D.B., MANDELIN II, A.M., GIORDANO, T., XUE, I., MALTER, J.S., SINGH, L.D., SNYDER, N.K. and SINGH, S.P. (1996). AUUUA-specific binding proteins in astrocytes. *Life Sci.58:* 2083-2089.

KANEDA, N., OSHIMA, M., CHUNG, S.Y. and GUROFF, G. (1992). Sequence of a rat *TIS11* cDNA, an immediate early gene induced by growth factors and phorbol esters. *Gene 118:* 289-291.

KIMMEL, C.B. and WARGA, R.M. (1987). Indeterminate cell lineage of the zebrafish embryo. *Dev. Biol.* 124: 269-280.

KIMMEL, C.B., BALLARD, W.W., KIMMEL, S.R., ULLMAN, B. and SCHILLING, T.F. (1995). Stages of embryonic development of zebrafish. *Dev Dynamics 203:* 253-310.

KIMMEL, C.B., WARGA, R.M. and SCHILLING, T.F. (1990). Origin and organization of the zebrafish fate map. *Development 108:* 581-594.

LAI, W.S., STUMPO, D.J. and BLACKSHEAR, P.J. (1990). Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. *J. Biol. Chem. 265:* 16556-16563.

LAI, W.S., THOMPSON, M.J., TAYLOR, G.A., LIU, Y. and BLACKSHEAR, P.J. (1995). Promoter analysis of *Zfp-36*, the mitogen-inducible gene encoding the zinc finger protein tristetraprolin. *J. Biol. Chem. 270:* 25266-25272.

MA, Q. and HERSCHMAN, H.R. (1995). The yeast homologue *YTIS11*, of the mammalian *TIS11* gene family is a non-essential glucose repressible gene. *Oncogene 10:* 487-494.

MA, Q., WADLEIGH, D., CHI, T. and HERSCHMAN, H. (1994). The *Drosophila TIS11* homologue encodes a developmentally controlled gene. *Oncogene 9:* 3329-3334.

MACDONALD, P.M. and SMIBERT, C.A. (1996). Translational regulation of maternal mRNAs. *Curr. Opin. Genet. Dev. 6:* 403-407.

MELLO, C.C., SCHUBERT, C., DRAPER, B., ZHANG, W., LOBEL, R. and PRIESS, J.R. (1996). The PIE-1 protein and germline specification in *C. elegans* embryos. *Nature 382:* 710-712.

MIZUNO, T., YAMAHA, E., KUROIWA, A. and TAKEDA, H. (1996). Mesoderm induction in zebrafish. *Nature 383:* 131-132.

NEUDECKER, T. (1976). Die Embryonalentwicklung des Karpfens (*Cyprinus carpio L.*) *Arch. Fisch. Wiss. 27:* 25-35.

NIE, X-F., MACLEAN, K.N., KUMAR, V., MCKAY, I.A. and BUSTIN, S.A. (1995). *ERF-2*, the human homologue of the murine *Tis11d* early response gene. *Gene 152:* 285-286.

PABO, C.O. and SAUER, R.T. (1992). Transcription factors: structural families and principles of DNA recognition. *Annu. Rev. Biochem. 61:* 1053-1095.

SCHULTE-MERKER, S., HO, R.H., HERRMANN, B.G. and NÜSSLEIN-VOLHARD, C. (1992). The protein product of the zebrafish homologue of the mouse T gene is expressed in nuclei of the germ ring and the notochord of the early embryo. *Development 116:* 1021-1032.

SHAW, G. and KAMEN, R. (1986). A conserved AU sequence from the 3'untranslated region of GM-CSF mRNA mediates selective mRNA degradation. *Cell 46:* 659-667.

SOLNICA-KREZEL, L., STEMPLE, D.L. and DRIEVER, W. (1995). Transparent things: cell fates and cell movements during early embryogenesis of zebrafish. *BioEssays 17:* 931-939.

STACHEL, S.E., GRUNWALD, D.J. and MEYERS, P.Z. (1993). Lithium perturbation and *goosecoid* expression identify a dorsal specification pathway in the pregastrula zebrafish. *Development 117:* 1261-1274.

STEVENS, C., SAMALLO, J., SCHIPPER, H., STROBAND, H.W.J. and TE KRONNIE, G. (1996a). Expression of *Hoxb-1* during gastrulation and segmentation stages of carp (*Cyprinus carpio*). *Int. J. Dev. Biol. 40:* 463-470.

STEVENS, C.J.M., TE KRONNIE, G., SAMALLO, J., SCHIPPER, H. and STROBAND, H.W.J. (1996b). Isolation of carp cDNA clones, representing developmentally-regulated genes, using a subtractive-hybridization strategy. *Roux Arch. Dev. Biol. 205:* 460-467.

STROBAND, H.W.J., STEVENS, C., TE KRONNIE, G., SAMALLO, J., SCHIPPER, H., KRAMER, B. and TIMMERMANS, L.P.M. (1995). Expression of carp *cdx1*, a *caudal* homolog, in embryos of the carp, *Cyprinus carpio. Roux's Arch. Dev. Biol. 204:* 369-377.

STROBAND, H.W.J., TE KRONNIE, G. and TIMMERMANS, L.P.M. (1996). Gastrulation in cyprinids: morphogenesis and gene expression. *Neth. J. Zool. 46:* 115-133.

STROBAND, H.W.J., TE KRONNIE, G. and VAN GESTEL, W. (1992). Differential susceptibility of early steps in carp (Cyprinus carpio) development to α-amanitin. *Roux Arch. Dev. Biol.* 202: 61-65.

TAYLOR, G.A., CARBALLO, E., LEE, D.M., LAI, W.S., THOMPSON, M.J., PATEL, D.D., SCHENKMAN, D.I., GILKESON, G.S., BROXMEYER, H.E., HAYNES, B.F., BLACKSHEAR, P.J. (1996[a]). A pathogenic role for TNFα in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. *Immunity 4:* 445-454.

TAYLOR, G.A., THOMPSON, M.J., LAI. W.S. and BLACKSHEAR, P.J. (1995). Phosphorylation of tristetraprolin, a potential zinc finger transcription factor, by mitogen stimulation in intact cells and by mitogen-activated protein kinase *in vitro. J. Biol. Chem. 270:* 13341-13347.

TAYLOR, G.A., THOMPSON, M.J., LAI, W.S. and BLACKSHEAR, P.J. (1996[b]). Mitogens stimulate the rapid nuclear to cytosolic translocation of tristetraprolin, a potential zinc finger transcription factor. *Mol. Endocrinol. 10:* 140-146.

THISSE, C., THISSE, B. and POSTLETHWAIT, J.H. (1995). Expression of *snail2*, a second member of the zebrafish *snail* family in cephalic mesendoderm and presumptive neural crest of wild-type and *spadetail* mutant embryos. *Dev. Biol. 172:* 86-99.

THISSE, C., THISSE, B., SCHILLING, T.F. and POSTLETHWAIT, J.H. (1993). Structure of the zebrafish *snail1* gene and its expression in wild-type, *spadetail* and *no tail* mutant embryos. *Development 119:* 1203-1215.

THOMPSON, M.J., LAI, W.S., TAYLOR, G.A. and BLACKSHEAR, P.J. (1996). Cloning and characterization of two yeast genes encoding members of the CCCH class of zinc finger proteins: zinc finger-mediated impairment of cell growth. *Gene 174:* 225-233.

TOYAMA, T., O'CONNELL, M.L., WRIGHT, C.V.E., KUEHN, M.R. and DAWID, I.B. (1995). *Nodal* induces ectopic *goosecoid* and *lim1* expression and axis duplication in zebrafish. *Development 121:* 383-391.

VARNUM, B.C., MA, Q., CHI, T., FLETCHER, B. and HERSCHMAN, H.R. (1991). The *TIS11* primary response gene is a member of a gene family that encodes proteins with a highly conserved sequence containing an unusual cys-his repeat. *Mol. Cell. Biol. 11:* 1754-1758.

VIGNALI, R., DE LUCCHINI, S., KABLAR, B., BARSACCHI, G. (1994). Genetic control of development in *Xenopus laevis*. *Genetica 94:* 235-248.

WARGA, R.M. and KIMMEL, C.B. (1990). Cell movements during epiboly and gastrulation in zebrafish. *Development 108:* 569-580.

WILSON, E.T., HELDE, K.A. and GRUNWALD, D.J. (1993). Something's fishy here - rethinking cell movements and cell fate in the zebrafish embryo. *Trends Genet 9:* 348-352.

WOLFFE, A.P. and MERIC, F. (1996). Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. *Int. J. Biochem. Cell Biol. 28:* 247-257.

WORMINGTON, M. (1993). Poly(A) and translation: development control. *Curr. Opin. Cell Biol. 5:* 950-954.

WORTHINGTON, M.T., AMANN, B.T., NATHANS, D. and BERG, J.M. (1996). Metal binding properties and secondary structure of the zinc-binding domain of Nup475. *Proc. Natl. Acad. Sci. USA 93:* 13754-13759.

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