Overlapping and distinct expression domains of *Zic2* and *Zic3* during mouse gastrulation

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Abstract

The *Zic* genes are the vertebrate homologues of the *Drosophila* Odd-paired gene. Mutations in two of these genes are associated with human congenital genetic disorders. Mutation of human and mouse *Zic2*is associated with holoprosencephaly which is caused by a defect of ventral forebrain development and mutation of human and mouse *Zic3* is associated with a X-linked heterotaxy syndrome that results from a failure of left-right axis formation. The embryological role of the *Zic* genes in these disorders is not well understood. Here we show that both of these genes are expressed prior to and throughout gastrulation. The genes show some broad similarities in their expression domains. Both genes however are also uniquely expressed in some tissues and these unique domains correlate with regions that potentially play a role in the aetiology of the respective genetic disorders. During primitive streak stages *Zic2* is expressed transiently and uniquely in the node and the head process mesendoderm. The head process is known to be required for the establishment or maintenance of the ventral forebrain, which is the region disrupted in holoprosencephaly. *Zic3* is not expressed in the node during primitive streak stages but is expressed in and around the node beginning from the head fold stages of development. This expression of *Zic3* correlates well with the first steps in the establishment of the left-right axis. We also examined the expression of the closely related gene, *Zic1*, and did not detect any transcripts in gastrulation stage embryos.

1. Results and discussion

Zic genes are the vertebrate homologues of the *Drosophila* pair-rule gene Odd-paired (*Opa*). In the mouse there are five *Zic* genes, currently named *Zic1-4* and *Opr* (Aruga et al., 1996a; Aruga et al., 1996b; Furushima et al., 2000). Each of the *Zic* genes contain five C2H2 zinc fingers and based on homology within the zinc finger domain *Zic1*, *2* and *3* are more similar to each other and appear to form a subfamily (Furushima et al., 2000). Of these three *Zic* genes, two have been implicated in human disease. Mutations in *ZIC2* are associated with holoprosencephaly and mutations in *ZIC3* with a X-linked heterotaxy (situs ambiguus) syndrome (Gebbia et al., 1997; Brown et al., 1998; Brown et al., 2001). The function of these genes appears to be conserved in the mouse because mutation of *Zic2* results in forebrain defects that include holoprosencephaly and mutation of *Zic3* causes left-right axis defects (Nagai et al., 2000; Elms et al., 2003, Carrel et al., 2000, Klootwijk et al., 2000; Purandare et al., 2002). However, the molecular manner in which each *Zic* gene functions in these processes remains unclear, for example no downstream Zic transcriptional targets have been identified and there is no clear evidence with regard to the signalling pathways that these genes respond to or interact with. Nor is the stage or tissue specificity of *Zic* gene function known.

One possibility is that the basis of these defects occurs during gastrulation. Holoprosencephaly occurs as a result of a failure to establish the ventral midline signalling centre in the forebrain (for review see Muenke and Beachy, 2000). It is known that *Zic2* is not expressed in the cells that constitute this signalling centre, indicating that any role of *Zic2* with respect to this forebrain signalling centre must be indirect. *Zic2* is however expressed in the dorsal forebrain and this expression is shifted ventrally in embryos that lack *Shh* (Brown et al., 2003). This demonstrates that *Zic2* may be a Shh responsive gene and could be involved in interpreting the Shh signal during forebrain development. An alternative possibility is that *Zic2* is required at some stage of the processes that establish the Shh secreting cells within the forebrain. In this case it may be

expected that *Zic2* would be expressed in one or other of the anterior midline tissues (head process mesendoderm and overlying neurectoderm) of the late gastrula that are the precursors of this centre (Camus et al., 2000). It is also likely that *Zic3* functions during gastrulation in its role of preventing heterotaxy. Components of the heterotaxy syndrome arise as a result of the failure to correctly establish the embryonic left-right axis (for review see Casey and Hackett, 2000). The specification of this axis begins during gastrulation and requires the presence of the midline tissues of node and notochord. Analysis of *Zic3* function in mouse and *Xenopus* embryos has shown that the likely site of *Zic3* function is in the node or its derivatives, rather than in the lateral plate mesoderm (Kitaguchi et al., 2000; Purandare et al., 2002).

Despite these predictions with regards the likely site of *Zic2* and *3* function, the expression pattern of these genes in the early post-implantation stage embryo is not well documented. While *Zic3* expression in the node has been reported (Purandare et al., 2002) the timing and location of this expression is not described. Additionally, Nagai et al (1997) show that *Zic* 2 and *3* are expressed during primitive streak stages by in situ hybridisation to sections of streak stage embryos, however the precise spatio-temporal pattern of *Zic2* and *Zic3* expression immediately prior to and during gastrulation is not known. An example of this lack of information is that a recent review describes *Zic*2 and *Zic*3 expression as commencing at gastrulation (Herman and El-Hodiri, 2002). In contrast we demonstrate that both genes are expressed at least as early as one and a half days prior to the initiation of gastrulation. Additionally, we demonstrate that *Zic2* is expressed in the head process mesendoderm and neurectoderm known to be required for the establishment and/or maintenance of the forebrain ventral midline. We also show that *Zic3* transcripts are detectable in the primitive streak but not in the node before the headfold stage of development, potentially pinpointing the developmental time at which *Zic3* first functions in the establishment of the left-right axis.

A further reason for detailing the expression pattern of these genes is to determine whether there is potential for functional redundancy between these closely related family members. The overlapping expression patterns of *Zic2* and *Zic3* do show that these genes may be able to compensate for one another in some cells of the mouse gastrula. To investigate this further we also examined the expression of the third member of this subfamily, *Zic1*. In contrast with the previously reported expression of this gene in sections of 7.0 and 7.5 dpc embryos (Nagai et al., 1997) we did not detect expression of this gene in wholemount specimens before the 4-somite stage of development.

1.1 Zic2 expression during gastrulation

Zic2 expression initiates prior to gastrulation when transcripts are localised to the ectoderm of both the extraembryonic and embryonic components of the egg cylinder at 5.5 dpc (Fig.1A). Transverse sections through this stage of embryo confirm that no *Zic2* expression is seen in the endoderm (Fig. 1B,C). This pattern of expression is maintained throughout the initial stages of gastrulation (Fig. 1E). When the first mesoderm emerges from the primitive streak it moves into the posterior extraembryonic region to form the posterior amniotic fold (paf) and this mesoderm does not express *Zic2* (Fig. 1F). Subsequently in late streak stage embryos, as the allantoic bud first appears and the node becomes visible, *Zic2* transcripts are found not only in the ectoderm but also in the primitive streak and in the emerging mesoderm in the distal two thirds of the embryonic region (Fig. 1G). Fig. 1D shows a section through the distal embryonic region of a late streak stage embryo. It can be seen that at this level *Zic2* is expressed throughout the ectoderm and mesoderm as well as in the cells of the primitive streak and the external most cells adjacent to the primitive streak. The external cells at this level of the primitive streak constitute the node. The transient expression of *Zic2* in the node continues until the late allantoic bud stage, and during these stages the midline mesendoderm immediately rostral of the node (known as the head process) also contains *Zic2* transcripts (Fig. 1H-J). However, signal is never seen in the

anterior definitive endoderm and likewise the anterior mesoderm does not express *Zic2* (Fig. 1J). Throughout the early allantoic bud stages the extraembryonic ectoderm continues to express *Zic2*. From the early allantoic bud stage on, *Zic2* expression in the embryonic ectoderm begins to gradually recede from the posterior most ectoderm. By the neural plate stage of development the posterior half of the embryo is essentially devoid of transcripts and *Zic2* signal is predominately seen in the neural plate region (Fig. 1K). At this stage as the headfolds begin to enlarge the neurectoderm continues to express *Zic2* although transcripts are already beginning to be depleted in the midline of the neural groove (data not shown). The expression in the extraembryonic ectoderm continues throughout these stages.

1.2 *Zic3* expression during gastrulation

Zic3 expression initiates prior to gastrulation with transcripts being seen throughout the extra embryonic ectoderm of 5.0 dpc stage embryos and within some epiblast cells (Fig. 2A). At all pre-gastrulation stages examined it was clear that the most distal epiblast cells do not express *Zic3* and that there is no expression in the endoderm. This was confirmed by sectioning of pre-gastrulation stage embryos (Fig. 2B-D). This pattern of expression is maintained up until the onset of gastrulation (Fig. 2E,F). As gastrulation initiates the expression in the ectoderm recedes from the anterior half of the embryo and *Zic3* is instead expressed in the region of the embryo that is lined by the mesoderm that emerges from the primitive streak to encircle the embryonic portion of the egg cylinder (compare 2E and 2G). The mesoderm that moves into the extra embryonic region does not contain *Zic3* transcripts, and the expression that can be seen at the posterior of the embryo at this stage is in the ectoderm of the posterior amniotic fold (arrow in Fig. 2G). In wholemount, mid-streak and full-streak stage embryos *Zic3* transcripts can be seen at the most anterior aspect of the embryo, at the junction of the embryonic and extraembryonic region. Sectioning of embryos revealed that this expression is in the anterior definitive endoderm (Fig. 2H,P). As the node forms at the anterior of the primitive streak it can be seen that, although the

ectoderm in this region contains transcripts, the node cells at the external surface of the embryo do not express *Zic3* (Fig. 2H). As gastrulation proceeds the pattern of *Zic3* expression remains relatively constant, with transcripts located in the wings of mesoderm of the embryonic region and in the primitive streak and ectoderm adjacent to the expressing mesoderm (Fig. 2I-K). The expression that can be seen in the posterior of the embryo (arrow in Fig. 2I,J) is located in the ectoderm. The mesoderm in this region is moving into the extraembryonic portion of the embryo and does not express *Zic3* as can be seen in the section shown in Fig. 2H. *Zic3* expression was not seen in the ventral node at any of these primitive streak stages, nor in the axial mesendoderm that emerges from the node.

As the headfolds develop *Zic3* expression is lost from the midline neurectoderm cells along the length of the axis, but continues throughout the rest of the neural plate (Fig. 2L and Q). In the cranial region the neurectoderm expression of *Zic3* begins to become regionalised with areas of lower signal including the prospective midbrain and future hindbrain (Fig. 2N). In early somite embryos the hindbrain contains one region with noticeably lower signal. This region is immediately caudal of the pre-otic sulcus that is known to mark the junction of future rhombomere 2 and 3 (Trainor and Tam, 1995), identifying it as prospective rhombomere 3. In later stage embryos when rhombomeres can be clearly distinguished it can be seen that *Zic3* transcripts are also down regulated in rhombomere 5 which was identified by the location of the otic sulcus that marks the boundary of rhombomere 5/6 (Trainor and Tam, 1995). Additionally at headfold stages *Zic3* transcripts disappear from the primitive streak but are now evident in a group of cells anterior to the primitive streak that constitute the node (Fig. 2M). This node expression continues, becoming more prominent in embryos with 7 somites (Fig. 2N, O and R) and is still present in embryos with 12 somites (data not shown). Inspection of the node expression in both wholemount specimens and in section failed to detect any evidence of asymmetric localisation of *Zic3* transcripts. During somite stages of development *Zic3* signal is

also detected in the paraxial mesoderm at the level of the node and more rostrally in the segmenting paraxial mesoderm (Fig. 2R and S).

The expression domains of *Zic2* and *Zic3* overlap in some respects. Prior to gastrulation both genes are expressed exclusively in the ectoderm. The only difference in their expression domains at this stage of development is that *Zic3* is never expressed in the distal most cells of the epiblast. As gastrulation proceeds both genes are expressed in the epiblast and in the newly formed mesoderm, however there are some differences in the expression domains. *Zic3* transcripts are seen transiently in the anterior most endoderm during the early streak stages of development while *Zic2* is never expressed in the anterior endoderm. Instead, *Zic2* is expressed at a more posterior level in the head process mesendoderm that emerges from the node during the early bud stage of development and underlies the prospective hindbrain neurectoderm. During the primitive streak stages *Zic3* transcripts are found in the ectoderm adjacent to the node but never in the cells that are passing through the node. This can be seen by comparing Fig. 1D and Fig. 2H. Both of these panels show a section through the node region of a full-streak stage embryo. *Zic2* is expressed in the external cells that lie lateral to the primitive streak whereas *Zic3* is not.

As neurulation proceeds the expression domains of *Zic2* and *Zic3* continue to share some broad characteristics; they are both expressed in the segmenting trunk paraxial mesoderm and the overlying neurectoderm. In both cases the expression in the neurectoderm begins to disappear from the future ventral midline and increasingly becomes restricted to the future dorsal neurectoderm. *Zic2* signal begins to be depleted in the future ventral midline neurectoderm at the neural plate stage. This is earlier than the loss of *Zic3* signal, which begins during headfold stages. Additionally, while *Zic2* transcripts are seen along the anterior-posterior length of the cranial neurectoderm, *Zic3* transcripts disappear from some hindbrain regions resulting in noticeably higher expression in prospective rhombomeres 2 and 4. A further difference is that

Zic3 is expressed throughout the node during the headfold and early somite stages of development, whereas the node expression of *Zic2* ceases before the headfold stage of development.

1.3 *Zic1* expression was not detected during gastrulation

Embryos ranging in stage from 5.5 dpc to 9.5 dpc were examined for *Zic1* expression. At stages later than 8.5 dpc (6-somites) *Zic1* expression was detected in the pattern previously described by Nagai et al (1997) (Fig.3 C and D). At gastrulation stages, however, we failed to detect *Zic1* signal (Fig. 3B), in contrast to Nagai and colleagues who used in situ hybridisation to formalin fixed, frozen sections to demonstrate a similar level of signal from all three *Zic* genes at primitive streak stages (Nagai et al., 1997). As described below in the discussion on probe specificity detection of *Zic1* signal generally required short reaction times (2 hours). To determine whether the apparent absence of *Zic1* transcripts during gastrulation was due to decreased sensitivity amongst this stage of embryos, for some gastrulation stage embryos the colourimetric reaction was allowed to continue for 24 hours. Although trapping of probe was seen in the embryonic cavities of these embryos, no signal was detected within the embryonic tissues (Fig.3B). These in situ hybridisation results were consistent over four replications of the experiment and with independent batches of probe, in each case the presence of later stage embryos in the same hybridisation chamber served as a positive control.

To further investigate the discrepancy between our results and those of Nagai and colleagues we used RT-PCR to investigate the expression of the *Zic* genes at 7.5 dpc and 9.5 dpc. Primers spanning intron 2/3 of *Zic1* and *Zic2* were used to amplify cDNA isolated from 7.5 dpc embryos or from 9.5 dpc embryos. As can be seen in Fig. 3A the ubiquitously expressed *Hprt* transcript could be amplified from both the 7.5 dpc and 9.5 dpc sample. In agreement with our in situ hybridisation results, *Zic2* transcripts could be amplified from both samples whereas under

identical conditions *Zic1* transcripts could only be amplified from the 9.5 dpc sample. In each reaction the major product corresponded to the predicted size of the product and the identity of the amplified material was confirmed by direct sequencing of PCR products. The RT-PCR result was confirmed using independent RNA samples. This data concurs with our in situ hybridisation results and we conclude that *Zic1* is not expressed during mouse gastrulation.

1.4 Probe specificity

Probes were initially generated to sequence verified clones that corresponded to the 3'UTR of *Zic1*, *Zic2* and *Zic3* and in situ hybridisation experiments performed on 9.5 dpc stage embryos. All probes replicated the 9.5 dpc expression patterns previously reported (Nagai et al., 1997). The three genes exhibit some distinct sites of expression at 9.5 dpc (for example *Zic3* is expressed in the tail bud and *Zic1* expression in the 9.5 dpc neural tube extends more ventrally). This demonstrates that the UTR probes do not cross hybridise to other *Zic* family members, as has been previously reported for Northern Hybridisation experiments using 3'UTR probes (Nagai et al., 1997). The *Zic1* probe reproducibly required shorter reaction times for signal development than did the *Zic2* and *Zic3* probes. We therefore designed additional probes to the coding sequence of *Zic2* and *Zic3*. These probes do not span the highly conserved Zinc finger domain but instead correspond primarily to the more divergent sequence 5' of the Zinc finger domains. In situ hybridisation experiments with these probes replicated the 9.5 dpc expression pattern observed with the respective 3'UTR probes, but required greatly decreased reaction times (30% - 50% of time required for signal detection with 3'UTR probes). For this reason, these probes were used for analysis of *Zic2* and *Zic3* expression.

2. Experimental methods and procedures

Mice were maintained in a light cycle of 12 hours light: 12 hours dark, the midpoint of the dark cycle being 1 AM. One PM on the day of appearance of the vaginal plug is designated 0.5 dpc.

Genetically identical embryos were collected from crosses between C3H/HeH females and 101/HeH males. All embryos were dissected from maternal tissue and Reichert's membrane removed in PBS with 10% newborn calf serum. The recovered embryos were staged according to Downs and Davies (1993). Embryos were transferred to 4% paraformaldehyde in PBS and after overnight fixation at 4ºC WMISH was carried out according to Wilkinson (1992) using the hybridisation conditions of Rosen and Beddington (1993). An antisense *Zic2* riboprobe was generated as described in Elms et al. (2003). For WMISH to *Zic3*, a cDNA probe corresponding to bases 646 – 1266 of mouse *Zic3* (Acc No.:NM_009575) was amplified and cloned into pGEM-Teasy (Promega). An antisense riboprobe was generated to this clone by *Nco* I digest and transcription with SP6 polymerase. For WMISH to *Zic1*, a cDNA probe was generated from a BMAP clone that contained bases 1948 – 2947 of mouse *Zic1* 3'UTR (NM_009573) cloned into pT7T3D-pac (Pharmacia). An antisense riboprobe was generated to this clone by digestion with *XhoI* and transcription with T3 RNA polymerase. After completion of the in situ procedure, embryos were destained in PBT (PBS with 0.1% Tween-20) for 48 hours and post-fixed in 4% paraformaldehyde in PBS for 1 hour at room temperature. Embryos were processed for photography through a glycerol series (50%, 80%, and 100%) and photographed in 100% glycerol on a glass slide. Before embedding for cryosectioning, embryos were returned to PBS and again post-fixed in 4% paraformaldehyde in PBS. The specimens were equilibrated overnight in 30% sucrose, then placed into OCT cyroembedding solution (BDH) and flash frozen in Liquid Nitrogen. 18 μm sections were cut using a Microm cryostat. Nomarski photographs were taken in a compound microscope (Zeiss) using tungsten film (Fuji 64T). For RT-PCR embryos were dissected as described above, washed in PBS and snap frozen on dry ice. For each 7.5 dpc sample, twenty late allantoic bud stage embryos were pooled and for each 9.5 dpc sample, three 21-23 somite stage embryos were pooled. Total RNA was isolated using a RNeasy Mini Kit (QIAGEN) and checked for integrity and quantified in RNA 6000 Nano Chips using a Bioanalyser 2100 (Agilent). cDNA was synthesised from 1 μg total RNA using a SMART PCR

cDNA synthesis Kit (Biosciences). The primers used for amplification and anticipated products sizes were as follows: *Zic1* 5': GCA AGA TGT GCG ATA AGT CC, *Zic1* 3': GGT TGT CTG TTG TGG GAG AC, product size 140 bp. *Zic2* 5': TCG TTG CGG AAG CAC ATG AA, *Zic2* 3': ACA GGT TGG AGC TGC TTT GT, product size 178 bp. The *Hprt* primers were as described in Nagamine et al. (1990). For direct sequencing of the RT-PCR products reactions were gel purified using the QIAquick Kit (QIAGEN). Sequencing was performed using the ABI Prism Big Dye Cycle Sequence Ready Reaction Kit (PE Biosystems) and reactions were separated on an ABI377 sequencer.

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Figure Legends

Fig. 1. *Zic2* expression during mouse gastrulation. In all pictures of embryos at stages where the anterior can be distinguished (after 6.0 dpc) the anterior is to the left and all intact embryos are shown in lateral view. (A) A 5.5 dpc, pre-streak stage embryo. (B) A transverse section through the extra embryonic region of a pre-streak stage embryo at the level shown in A. (C) A transverse section through the embryonic region of a pre-streak stage embryo at the level shown in A. (D) A transverse section through the embryonic region of a 7.0 dpc, late-streak stage embryo at the level shown in G. The primitive streak and node are to the right. (E) A 6.5 dpc, early streak stage embryo at the onset of gastrulation. (F) A 6.75 dpc mid-streak stage embryo with mesoderm moving into the posterior amniotic fold. (G) A 7.0 dpc late-streak stage embryo. The node is now visible and *Zic2* transcripts are seen in the node. (H) A 7.25 dpc early allantoic bud stage embryo. (I) A 7.5 dpc late allantoic bud stage embryo. Expression in the node and emerging head process is still seen. (J) A longitudinal section through a 7.5 dpc embryo such as that shown in I. *Zic2* transcripts are not found in the mesoderm of the extra embryonic region or of the proximal embryonic region. (K) A 7.75 dpc early headfold stage embryo. *Zic2* transcripts are now mainly confined to the anterior half of the embryo and the expression in the node has ceased. ae: anterior definitive endoderm, am: anterior mesoderm, hp: head process, n: node, paf: posterior amniotic fold. Scale bar, 50 μm (A-D), 100 μm (E-G), 200 μm (H, I and K) and 170 μm (J).

Fig. 2. *Zic3* expression during mouse gastrulation. In all pictures of intact embryos at stages where the anterior can be distinguished (after 6.0 dpc) anterior is to the left (except L and M), in L and M anterior is to the top. Anterior is to the left in the sections shown in H and P. The intact embryos in A-K are shown in lateral view and those in L-O are shown in dorsal view. (A) A 5.0 dpc, pre-streak stage embryo. (B) A transverse section through the extra-embryonic region of a pre-streak embryo at the level shown in E. (C) A transverse section through the proximal embryonic region of a pre-streak stage embryo at the level shown in E. (D) A transverse section

through the distal embryonic region of a pre-streak stage embryo at the level shown in E. (E) A 6.0 dpc pre-streak stage embryo. (F) A 6.25 dpc embryo just prior to the onset of gastrulation. (G) A 6.75 dpc, mid-streak stage embryo, showing the formation of the posterior amniotic fold. (H) A longitudinal section through the 7.0 dpc full-streak stage embryo shown in I. (I) A 7.0 dpc full-streak stage embryo. (J) A 7.25 dpc early allantoic bud stage embryo. (K) A 7.75 dpc early headfold stage embryo. (L) A flatmount of the trunk neurectoderm of a headfold, pre-somite stage embryo showing that transcription has diminished in the midline neurectoderm. (M) The posterior of a headfold, pre-somite stage embryo with transcripts in the neurectoderm and at the edge of the node. (N) A 7-somite stage embryo, note the regionalisation of expression in the cranial neurectoderm. (O) A higher power view of the posterior of a 7-somite stage embryo in which some transcripts are visible at the edge of the node and some signal is seen within the node itself. (P) A transverse section through the embryonic portion of a 6.75 dpc, mid-streak stage embryo at the level shown in G. (Q) A transverse section through the trunk of a 1-somite stage embryo. (R) A transverse section through a 7-somite embryo at the level of the node. (S) A transverse section through the trunk of a 7-somite embryo anterior of the node. mn: midline neurectoderm, r3: prospective rhombomere 3, n: node. Scale bar, 50 μm (A), 90 μm (B-D), 100 μm (E-G), 150 μm (H-M and O), 300 μm (N), 170 μm (P), 120 μm (Q-S).

Fig. 3. *Zic1* is not expressed at gastrulation. In all pictures of embryos the anterior is to the left and the embryos are shown in lateral view. (A) RT-PCR amplification of *Zic1* and *Zic2* transcripts from 7.5 dpc and 9.5 dpc embryos. The samples are as follows: M, 1Kb+ size marker, the two smallest bands correspond to 100 bases and 200 bases respectively; 1, *Zic1* primers and 7.5 dpc cDNA; 2, *Zic1* primers and 9.5 dpc cDNA; 3, *Zic1* primers and no cDNA; 4, *Zic2* primers and 7.5 dpc RNA; 5, *Zic2* primers and 9.5 dpc cDNA; 6, *Zic2* primers and no cDNA; 7, *Hprt* primers and 7.5 dpc cDNA; 8, *Hprt* primers and 9.5 dpc cDNA; 9, *Hprt* primers and no cDNA. (B) A 7.0 dpc full-streak stage embryo. The signal in the extraembryonic region is due to probe

trapping. (C) A 6-somite stage embryo. (D) A 20-somite stage embryo. Scale bar, 100 μm (B), 200 μm (C) and 400 μm (D).

References

Aruga, J., Nagai, T., Tokuyama, T., Hayashizaki, Y., Okazaki, Y., Chapman, V.M. and Mikoshiba, K. (1996a) The mouse zic gene family. Homologues of the Drosophila pair-rule gene odd-paired. J Biol Chem 271, 1043-7.

Aruga, J., Yozu, A., Hayashizaki, Y., Okazaki, Y., Chapman, V.M. and Mikoshiba, K. (1996b) Identification and characterization of Zic4, a new member of the mouse Zic gene family. Gene 172, 291-4.

Brown, S.A., Warburton, D., Brown, L.Y., Yu, C.Y., Roeder, E.R., Stengel-Rutkowski, S., Hennekam, R.C. and Muenke, M. (1998) Holoprosencephaly due to mutations in ZIC2, a homologue of Drosophila odd-paired. Nat Genet 20, 180-3.

Brown, L.Y., Odent, S., David, V., Blayau, M., Dubourg, C., Apacik, C., Delgado, M.A., Hall, B.D., Reynolds, J.F., Sommer, A., Wieczorek, D., Brown, S.A. and Muenke, M. (2001) Holoprosencephaly due to mutations in ZIC2: alanine tract expansion mutations may be caused by parental somatic recombination. Hum Mol Genet 10, 791-6.

Brown, L.Y., Kottmann, A.H. and Brown, S. (2003) Immunolocalization of Zic2 expression in the developing mouse forebrain. Gene Expr Patterns 3, 361-7.

Camus, A., Davidson, B.P., Billiards, S., Khoo, P., Rivera-Perez, J.A., Wakamiya, M., Behringer, R.R. and Tam, P.P. (2000) The morphogenetic role of midline mesendoderm and ectoderm in the development of the forebrain and the midbrain of the mouse embryo. Development 127, 1799-813.

Carrel, T., Purandare, S.M., Harrison, W., Elder, F., Fox, T., Casey, B. and Herman, G.E. (2000) The X-linked mouse mutation Bent tail is associated with a deletion of the Zic3 locus. Hum Mol Genet 9, 1937-42.

Casey, B. and Hackett, B.P. (2000) Left-right axis malformations in man and mouse. Curr Opin Genet Dev 10, 257-61.

Downs, K.M. and Davies, T. (1993) Staging of gastrulating mouse embryos by morphological landmarks in the dissecting microscope. Development 118, 1255-66

Elms, P., Siggers, P., Napper, D., Greenfield, A. and Arkell, R. (2003) *Zic2* is required for neural crest formation and hind brain patterning during mouse development. Dev Biol 264, 391-406.

Furushima, K., Murata, T., Matsuo, I. and Aizawa, S. (2000) A new murine zinc finger gene, Opr. Mech Dev 98, 161-4.

Gebbia, M., Ferrero, G.B., Pilia, G., Bassi, M.T., Aylsworth, A., Penman-Splitt, M., Bird, L.M., Bamforth, J.S., Burn, J., Schlessinger, D., Nelson, D.L. and Casey, B. (1997) X-linked situs abnormalities result from mutations in ZIC3. Nat Genet 17, 305-8.

Herman, G.E. and El-Hodiri, H.M. (2002) The role of ZIC3 in vertebrate development. Cytogenet Genome Res 99, 229-35.

Hitchins, M.P., Bentley, L., Monk, D., Beechey, C., Peters, J., Kelsey, G., Ishino, F., Preece, M.A., Stanier, P. and Moore, G.E. (2002) DDC and COBL, flanking the imprinted GRB10 gene on 7p12 are biallelically expressed. Mamm Genome 12, 686-691.

Kitaguchi, T., Nagai, T., Nakata, K., Aruga, J. and Mikoshiba, K. (2000) Zic3 is involved in the left-right specification of the Xenopus embryo. Development 127, 4787-95.

Klootwijk, R., Franke, B., van der Zee, C.E., de Boer, R.T., Wilms, W., Hol, F.A. and Mariman, E.C. (2000) A deletion encompassing Zic3 in bent tail, a mouse model for X-linked neural tube defects. Hum Mol Genet 9, 1615-22.

Muenke, M. and Beachy, P.A. (2000) Genetics of ventral forebrain development and holoprosencephaly. Curr Opin Genet Dev 10, 262-9.

Nagai, T., Aruga, J., Takada, S., Gunther, R.S., Schughart, K. and Mikoshiba, K. (1997) The expression of the mouse Zic1, Zic2 and Zic3 gene suggests an essential role for Zic gene in body pattern formation. Dev. Biol. 182, 299-313.

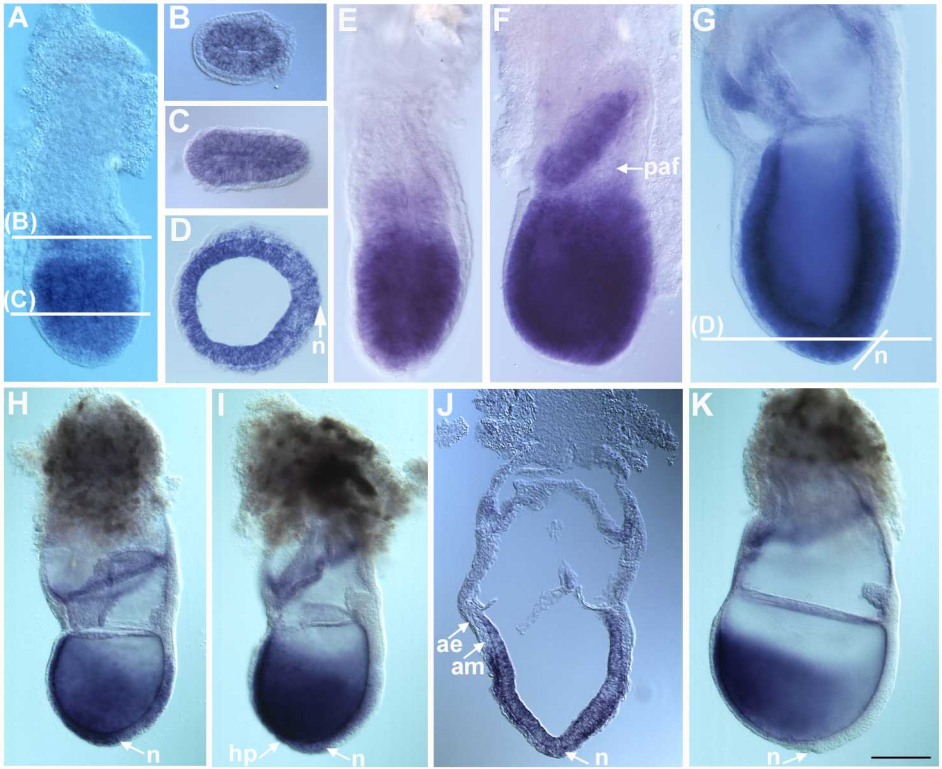
Nagai, T., Aruga, J., Minowa, O., Sugimoto, T., Ohno, Y., Noda, T. and Mikoshiba, K. (2000) Zic2 regulates the kinetics of neurulation. Proc Natl Acad Sci U S A 97, 1618-23.

Purandare, S.M., Ware, S.M., Kwan, K.M., Gebbia, M., Bassi, M.T., Deng, J.M., Vogel, H., Behringer, R.R., Belmont, J.W. and Casey, B. (2002) A complex syndrome of left-right axis, central nervous system and axial skeleton defects in Zic3 mutant mice. Development 129, 2293-302.

Rosen, B. and Beddington, R.S.P. (1993) Whole-mount *in situ* hybridization in the mouse embryo: gene expression in three dimensions. Trends Genet. 9, 162-167.

Trainor, P.A. and Tam, P.P.L. (1995) Cranial paraxial mesoderm and neural crest clls of the mouse embryo: co-distribution in the craniofacial mesenchyme but distinct segregation in branchial arches. Development 121, 2569-2582.

Wilkinson, D.G. (1992) Whole mount in situ hybridisation of vertebrate embryos. In Wilkinson, D.G. (ed.), In situ hybridisation, IRL Press, Oxford, Practical Approach Series, pp. 75-83.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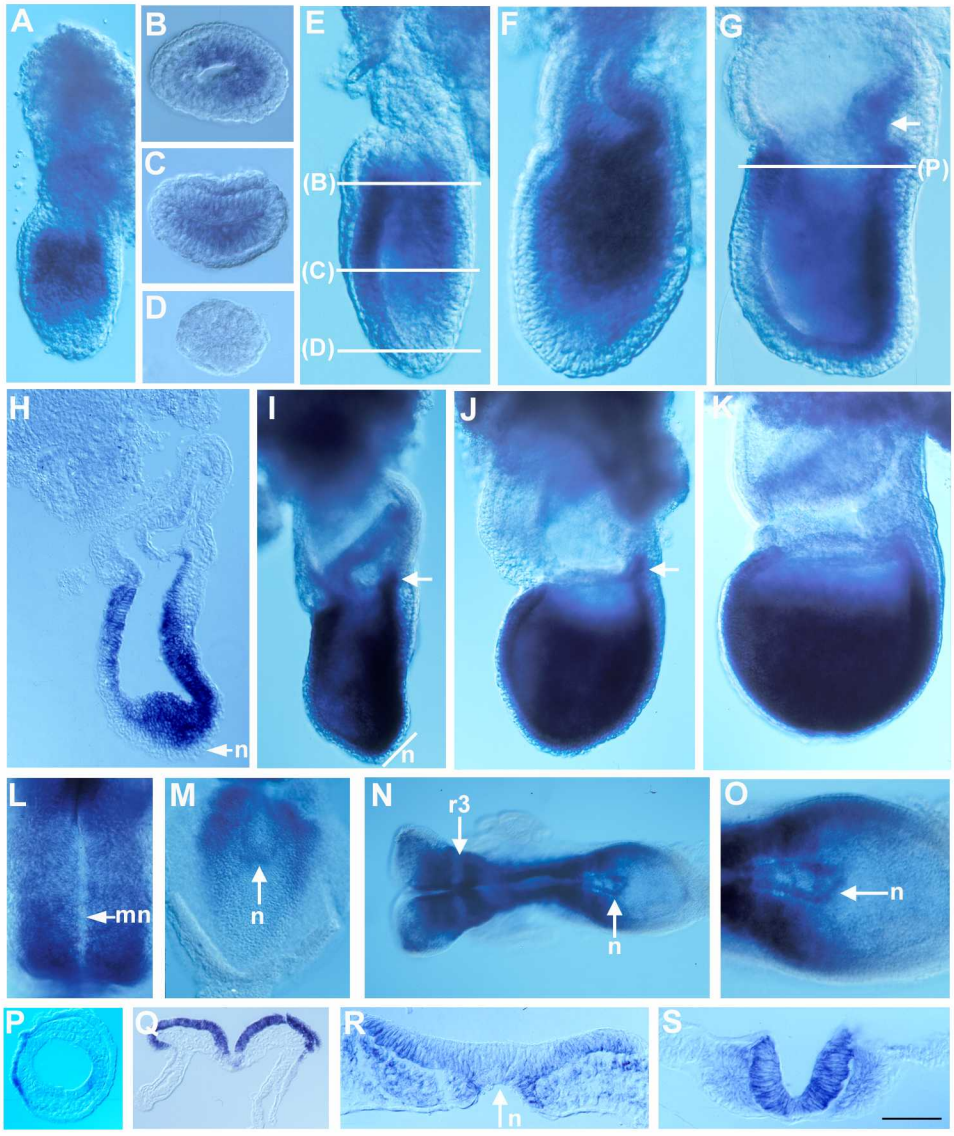
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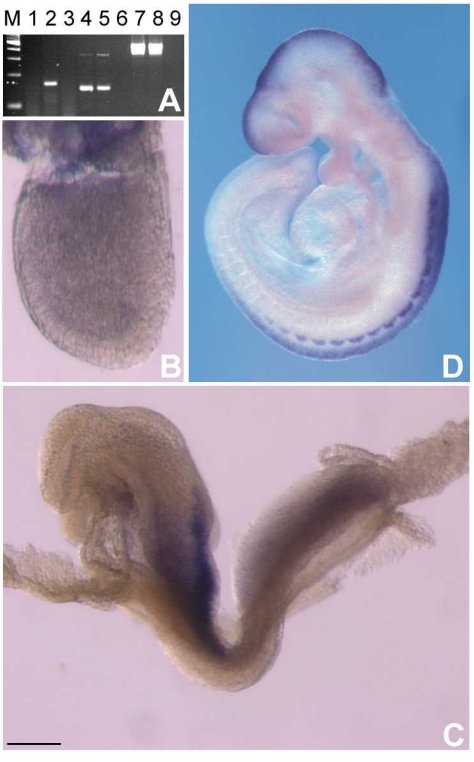
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