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### Genomes & Developmental Control

# Zebrafish *gcm2* is required for gill filament budding from pharyngeal ectoderm

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#### Abstract

The pharyngeal arches give rise to multiple organs critical for diverse processes, including the thymus, thyroid and parathyroids. Several molecular regulators of thymus and thyroid organogenesis are strikingly conserved between mammals and zebrafish. However, land animals have parathyroids whereas fish have gills. The murine transcription factor Glial cells missing 2 (Gcm2) is expressed specifically in the parathyroid primordium in the endodermal epithelium of the third pharyngeal pouch, and in both mice and humans is required for normal development of parathyroid glands. The molecular regulation of fish gill organogenesis remains to be described. We report the expression of gcm2 in the zebrafish pharyngeal epithelium and a requirement for Hox group 3 paralogs for gcm2 expression. Strikingly, zebrafish gcm2 is expressed in the ectodermal portion of the pharyngeal epithelium and is required for the development of the gill filament buds, precursors of fish-specific gill filaments. This study identifies yet another role for a GCM gene in embryonic development and indicates a role for gcm2 during the evolution of divergent pharyngeal morphologies.

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#### Introduction

Comparison of the embryology of pharyngeal organogenesis in land animals and fish reveals several striking similarities despite significant differences in the final anatomical outcome. The mammalian pharyngeal arches give rise to the thymus, thyroid and parathyroid glands. Fish also develop thyroid and thymus glands from their pharyngeal regions, but lack parathyroid glands and form gills. Whilst recent studies have shown that many key molecular regulators of thymus and thyroid organogenesis are functionally conserved between the zebrafish and mammals (Boehm et al., 2003; Elsalini et al., 2003; Rohr and Concha, 2000; Wendl et al., 2002), the molecular regulation of gill organogenesis and its relationship, if any, to mammalian pharyngeal organogenesis, remains to be described.

In both mice and zebrafish, the thyroid develops from the ventral midline endodermal epithelium of the pharynx (Elsalini et al., 2003; Macchia, 2000; Rohr and Concha, 2000; Wendl et al., 2002). The early thyroid primordium expresses *hhex* and *nkx2.1* in both mice and zebrafish and furthermore, the loss of function phenotypes for these early thyroid determinants are similar, indicating significant conservation of both thyroid morphogenesis and its molecular control (Elsalini et al., 2003; Rohr and Concha, 2000). The murine thymus develops from the thymic primordium in the endoderm of the third pharyngeal pouch. Prior to differentiation towards definitive thymic tissues, thymic

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primordium is marked by the transcription factor *Foxn1* (the *nude* gene), which is expressed in the portion of the third pharyngeal arch primordium immediately ventral to and abutting the parathyroid primordium (Gordon et al., 2001). Loss of *Foxn1* leads to the ablation of the ablation of thymus in the classic *nude* mouse (Blackburn et al., 1996; Nehls et al., 1996). In zebrafish, *foxn1/whnb* expression is also restricted to the pharyngeal epithelium (in the fifth pharyngeal [i.e., third branchial] arch), where its appearance precedes the expression of functional thymic markers such as *rag1* and *lck* (Hansen and Zapata, 1998; Schorpp et al., 2002; Willett et al., 1997).

Prior to organogenesis, the segmentation and patterning of the pharyngeal pouches requires the activity of multiple Hox genes in both mammals and in zebrafish. HoxA3 is critical for the establishment of the murine parathyroid/ thymic primordium in the third pharyngeal pouch, and HoxA3-deficient mice lack both a thymus and parathyroids (Manley and Capecchi, 1995, 1998; Su et al., 2001). In HoxA3-deficient mice, the loss of thymic marker Pax1 and parathyroid marker Gcm2 occurs from as early as E10.5 in an otherwise normal developmental context, and so is considered a likely cell autonomous requirement in pharyngeal endoderm, rather than due to neural crest defects (Manley and Capecchi, 1995; Su et al., 2001). Other Hox group 3 paralogs have also been implicated in pharyngeal organ development, with HoxA3+/-, HoxB3-/-, HoxD3 - /- compound mutants displaying multiple pharyngeal defects (Manley and Capecchi, 1998). Zebrafish hoxa3 and hoxb3 have almost identical expression patterns to their murine orthologs, but no role in pharyngeal organogenesis has yet been reported (Hadrys et al., 2004; Prince et al., 1998; Schilling et al., 2001).

Given the degree of conservation in the molecular pathways required for early pharyngeal patterning and thyroid and thymus organogenesis, we were curious to understand the molecular processes responsible for one striking difference between the mammalian and piscine pharyngeal regions—the presence of parathyroid glands only in land animals, and of gills only in fish. The evolutionary origins of the parathyroid glands are coincident with the transition of marine vertebrates onto land, a process in which there was no longer a requirement for gills. The most basal existing taxa with parathyroid glands are amphibia, and all modern fish lack parathyroid glands (Hoar and Randall, 1969).

In mice, the parathyroid glands develop as evaginations from the third pharyngeal pouch endoderm, and in humans, as evaginations from the third and fourth pharyngeal pouches. In mammals, the transcription factor *Gcm2* (Glial cells missing 2) is expressed in the pharyngeal parathyroid primordium and later in the derived parathyroid glands (Gordon et al., 2001; Gunther et al., 2000; Kim et al., 1998). *Gcm2*-deficient gene targeted mice lack parathyroids (Gunther et al., 2000) and *Gcm2* has been exploited as a defining marker of parathyroid primordium in murine development (Correa et al., 2002; Gordon et al., 2001; Su et al., 2001; Xu et al., 2002). Furthermore, a human family carrying a deletion in the *GCM2* gene displays familial hypoparathyroidism (Ding et al., 2001), characterising mammalian *GCM2* as a conserved early regulator of parathyroid gland development from pharyngeal endoderm.

In this study, we have investigated the function of zebrafish gcm2. We show that gcm2 is expressed in the ectodermal epithelium of pharyngeal arches 3–6 (the branchial arches) and in the early gill filament evaginations as they bud from this region. Interestingly, expression of gcm2 is dependent on normal specification of the adjacent pharyngeal endoderm. We also show that zebrafish Hox group 3 paralogs are required for gcm2 expression, similar to murine HoxA3 and Gcm2 (Su et al., 2001). When gcm2 function is blocked by morpholino-mediated knockdown of translation, the gill filaments fail to bud. Our studies suggest that although gills and parathyroids serve different physiological functions, and derive from different germ layers, their development employs conserved molecular regulators.

#### Materials and methods

#### Zebrafish

Wild-type and *cloche* mutant zebrafish stocks were held in the Ludwig Institute for Cancer Research Aquarium Facility using standard husbandry techniques. All procedures on live animals and embryos were approved by the Ludwig Institute for Cancer Research Animal Ethics Committee.

Collected embryos were incubated at 28°C on a plate warmer; those for analysis at later timepoints were maintained in eggwater containing 0.003% 1-phenyl-2-thiourea (PTU) from 12 h post-fertilisation (hpf). Mutant embryos were sourced as follows: fixed *casanova* embryos a gift from Didier Stainier (University of California, San Francisco, CA), fixed *foxi1* embryos a gift from Nancy Hopkins and Adam Amsterdam (Massachusetts Institute of Technology, Cambridge, MA), fixed *swirl* and maternal zygotic *one eyed pinhead* embryos a gift from Robert Ho (University of Chicago, Chicago, IL).

#### Cloning of gcm2

Degenerate oligonucleotide primers were designed employing the CODEHOP method (Rose et al., 1998), primers were: 5'-GGATGGGCCATGAGAAACAC-NAAYAAYCA-3' and 5'-TCTGCTGCTTCTGTC-TGGCYTTRTCRCA-3'. The resultant 155-bp product was subcloned into pCR2.1 (Invitrogen) and the sequence used to design oligonucleotide primers for 5' and 3' RACE (rapid amplification of cDNA ends), primers were: 5'-GTCTCAG-CTGAAGTTTGGATCCGTCTGG-3' and 5'-TCTTG- TCTTGGTGTGGTGGTGGTGTGCTCAC-3'. Products were cloned into pCR2.1 (Invitrogen) and sequenced.

A PolyA Tract mRNA purification system IV (Promega) was used to isolate high quality polyA mRNA for use as a RACE template. RACE was performed using the CLON-TECH SMART<sup>™</sup> RACE cDNA Amplification Kit to the manufacturer's specifications.

The *gcm2* cDNA was PCR amplified from RACE template (VENT DNA polymerase (New England Biolabs)) and cloned into pCS2+ (*ATGgcm2*) (Turner and Weintraub, 1994) (*Eco*RI, *Xho*I), primers were: 5'-GCGCGAATTCGC-CACCATGTCCAAATCCTCAGATCAGTTTGAC-3' and 5'-GCGCCTCGAGTCAGTCAGTATTCCCCGCTGTCA-TATCTG-3'. The *UTRgcm2* cDNA construct was created by subcloning a region including 5' UTR from the 5' RACE product (*Eco*RI, *Pvu*II) into the *gcm2* cDNA construct in pCS2+.

The phylogenetic tree (Fig. 1a) was generated as previously described (Lieschke et al., 2002). The genomic Southern blot was performed using the cloned CODEHOP PCR product as a probe as previously described (Lieschke et al., 2002). Stringency was defined by final washing in  $0.5 \times$  SSC, 1% SDS at 56°C.

#### RT-PCR analysis

RT-PCR was performed as previously described (Lieschke et al., 2002). Primer sequences were: gcm2 5'-GATC-CAAATCATGTCCAAATCCTC-3' and 5'-AGCCTTCCCA-TCTACTCTCC-3';  $\beta$ -actin 5'-TGGCATCACACCTTCTAC-3' and 5'-AGACCATCACCAGAGTCC-3'.

#### Whole-mount in situ hybridisation

Whole mount in situ hybridisation analyses were performed as previously described (Lieschke et al., 2002; Oates et al., 1999). Two gcm2 riboprobe templates were generated by subcloning the 5' RACE product (*XhoI*, *Eco*RI; Probe 1) and a 3' fragment cut out of the cloned cDNA (*Eco*RV, *XhoI*; Probe 2) into pBluescript (Stratagene). Riboprobes were synthesised by linearising and transcribing with: Probe 1 *XhoI*, T3 for antisense, *Eco*RI, T7 for sense and Probe 2 *Eco*RV, T3 for antisense. Sense controls produced no staining in initial in situ hybridisation experiments and so were not repeated routinely.

#### mRNA synthesis

The *gcm2* mRNA was made by linearising template constructs with *Not*1 followed by synthesis of capped full-length mRNA as previously described (Lieschke et al., 2002). The full-length cDNAs for *hoxa3* and *hoxb3* (accession numbers: *hoxa3* NM\_131534, and *hoxb3* AJ537509) were directly amplified from an 18 hpf cDNA library (kindly provided by Bruce Appel, Vanderbilt

University, Nashville, TE) and cloned into pCS2+, *hoxa3* as an *XhoI–XbaI* fragment and *hoxb3* as an *EcoRI–XbaI* fragment. Primers used were: *hoxa3* 5'-CCGCTCGAGG-GAAACGGCGAGATGCAAAAG-3' and 5'-GCTCTAGAACTGCGCCACTATAAATGCGTC-3'; *hoxb3* 5'-GGAATTCAGCAACTCGATTTTTGGAAA-3' and 5'-GCTCTAGATGCCACTGTTACCCTGTAGC-3'. mRNA for in vitro translation (Fig. 7) was synthesised as previously described (Hunter and Prince, 2002).

#### Morpholino oligonucleotides

Morpholino oligonucleotides (MOs) used were synthesised by Genetools, LLC. Microinjection was performed as previously described (Lieschke et al., 2002).

Sequences for targeting *gcm2* were (with start codon underlined): M01, 5'-CTGATCTGAGGATTTGGA<u>CAT</u>-GATT-3'; M02, 5'-AGTTTCAAGGCAAAATTCAGC-TCGG-3'; M03, 5'-TGGA<u>CAT</u>GATTTGAGTCAACTC CGG-3' and M01 control with five mismatched base pairs (lower case), 5'-CTcATgTGAGGAaTTGGACtTGtTT-3'.

gcm2 morpholinos were injected in the concentration range 3.75–15 ng/embryo: 7.5 ng/embryo and 15 ng/embryo produced the typical gcm2 morphant phenotype described. A total morpholino concentration of 7.5 ng/embryo did not produce common non-specific defects (Nasevicius and Ekker, 2000) and was selected for these studies as a maximum dose deliverable to theoretically maximise the duration and efficiency of translational interference.

Morpholino sequences for targeting *hoxa3* and *hoxb3* were: Start codon targeting morpholinos; hoxa3MO, 5'-CAGTAGGTTGCCTTTTGCATCTCGC-3' and hoxb3MO, 5'-CGTAGTAGGTCGTTTTCTGCATTTC-3' hoxa3MO and hoxb3MO morpholinos were injected at a concentration of 4 ng/embryo (4 + 4 ng/embryo for combined morpholino injections) which failed to produce common non-specific defects. Hox group 3 start codon targeting morpholino knockdown data was also confirmed with independent hoxa3 and hoxb3 morpholino oligonucleotides targeted to the first donor splice site within each gene. The splice site directed morpholino sequences were: hoxa3MO<sup>Sp</sup>, 5'-GGTTGATG-TAATCACCTGAAATGAT-3' and hoxb3MOSp, 5'-TTTGGCAAACACACCATTAGCTGAG-3'. hoxa3MO<sup>Sp</sup> and hoxb3MO<sup>Sp</sup> were injected at 4 ng/embryo (4 + 4 ng/ embryo for combined morpholino injections). The standard random sequence morpholino control used was: 5'-CCTCTTACCTCAGTTACAATTTATA-3'.

#### Inhibition of in vitro translation assays

In vitro translation of mRNA was performed using Promega's Rabbit Reticulolysate System according to manufacturer's instructions in the presence or absence of a MO (1:100 dilution final from a 20-mg/ml stock). Electrophoresis was performed using a 12% Tris–Glycine precast gel (Invitrogen), and transferred to a nylon



Fig. 1. Cloning of zebrafish *gcm2*. (a) Unrooted phylogenetic tree of Glial cells missing family members in alignment below (b). *Danio rerio* (zebrafish) gene is boxed and falls into the *gcm2* clade. Bootstrap values are shown at nodes. Human (hs), Mouse (mm), Rat (rn), Zebrafish (dr), Fugu (tr), *Tetraodon* (tn), *Xenopus* (xl) and *Drosophila* (dm). (b) Multiple sequence alignment of Glial cells missing family members, spanning the DNA binding domain. Alignment was generated using the conserved DNA binding sequences only (from "WDIND" to "EARR" sequence). *Danio rerio* sequence is indicated by an arrow. (c) Depiction of the cDNA and genomic structure of the zebrafish *gcm2* gene. Intron sequences were elucidated from the Sanger Centre trace sequence repositories and aligned contig sequences. Sequence analysis identified the start codon at 359 bp and identified an ORF of 1488 bp encoding a 496-amino acid protein. The *gcm2* intron structure is conserved compared with the mouse and human genes, which also contain four small introns in the 5' coding sequence including sequence encoding the conserved N-terminal DNA binding domain, which spans the first four exons. The coding sequence is shown in light grey, the conserved DNA binding domain in dark grey, the untranslated region (UTR) in white and the polyA tail in black. (d) Genomic Southern blot using sequences corresponding to the *gcm2* DNA binding domain as a probe. Restriction enzymes used are indicated and size bars indicate fragment size in kilobases.

membrane (Hybond N+, Amersham Pharmacia Biotech) by standard methods. The membrane was exposed overnight to a Phosphor screen (Molecular Dynamics Inc.) and processed in a Molecular Dynamics Phosphorimager system; single protein bands of the expected sizes were produced from each RNA. Relative protein amounts were quantified using the ImageQuant Program version 1.2 (Molecular Dynamics Inc.).

#### Staining of craniofacial cartilage and bone

Morphant embryos for Alcian blue staining were preselected for the typical morphological phenotype described at 4 and 5 dpf. Anesthetised larvae were fixed in 3.7% formaldehyde at room temperature for several hours to overnight, they were rinsed briefly in acid alcohol solution (70% ethanol, 1% concentrated HCl) and then transferred



Fig. 2. Embryonic expression of zebrafish gcm2. (a, b, c) Whole mount in situ hybridisation detected gcm2 transcripts in the pharyngeal arches. Transcripts were detected in arches 3–6 at 50 hpf and by 96 hpf were present in distinct buds at the surface of the arches. Lateral view of 50 hpf embryo (a) showing expression in arches 3–6. Ventral view of 96 hpf embryo (b) showing expression specific to the developing gill arches and not impinging on the ventral midline. Ventro-lateral view of the left gill arches (c) showing expression of gcm2 in gill filament buds (arrowhead). (d, e) Sections through the pharyngeal arches of embryos post in situ hybridisation for gcm2. At 76 hpf (d) and 96 hpf (e), expression was restricted to the pharyngeal external (lateral) ectodermal epithelium surrounding arches 3–6 (labelled). Rostral is to the left of the image and medial to the top, Y = yolk. Scale bars indicate 50 µm. (f–h) Whole mount in situ hybridisation detected gcm2 expression in a population of superficial epidermal cells. Expression of gcm2 in dispersed cells (arrowhead) at 12 hpf (f) and 20 hpf (g). (h) Cross-section demonstrating that the gcm2 expressing cells (arrowhead) are immediately sub epithelial (Y = yolk, scale bar = 50 µm). (i) RT-PCR detection of gcm2 transcripts during zebrafish embryogenesis and in adult tissues. cDNA templates for lanes (left to right) are 2–24 hpf (staged every 2 h), 48 and 72 hpf staged embryos; testis (T); brain (B); eggs and ovaries (O); muscle (M); crude skin and adipose extract (I); spleen (Sp); liver (L); airbladder (A); kidneys (K); genomic DNA (gDNA) and water (H<sub>2</sub>0).  $\beta$ -actin control is shown below. gcm2 transcripts were first detected in 10 hpf cDNA, and throughout development to 72 hpf. Expression of gcm2 was also detected in cDNA from adult brain, testis and a crude skin and adipose tissue extract. The primers spanned introns and amplified larger fragments from gDNA.

into a 0.1% Alcian blue solution in 80% ethanol/20% glacial acetic acid. After overnight staining, larvae were rinsed in ethanol, cleared in acid alcohol solution and imaged in 50% glycerol, 0.5% KOH. Calcein staining of early larval bones was performed as previously described (Du et al., 2001).

#### Microscopy and imaging

Embryos were imaged using an Olympus DP10 digital camera on either a Leica FL-111 dissecting microscope or a NIKON eclipse TE2000-E compound microscope. DIC microscopy was performed on a NIKON eclipse TE2000-E compound microscope. Calcein staining false colour images were created on a Leica FL-111 dissecting microscope with a Coolsnap HQ camera (Roper Scientific Photometrics) using RS image 1.7.3 software (Roper Scientific). To optimise clarity in the presentation of the panels, rostral is left and dorsal is up in all panels unless otherwise indicated, scale bars are provided only in the absence of standard anatomical landmarks; the inter-pharyngeal arch distance, indicated precisely in Figs. 2d and e, demonstrates the magnification.

#### Results

#### Cloning of gcm2

We cloned and examined gcm2 in zebrafish. A 2116 base pair (bp) zebrafish gcm cDNA was isolated (Accession number AY217729). The cDNA encoded a predicted 496 amino acid protein 45% identical to human GCM2, and 42% identical to mouse Gcm2 across the full length of the predicted protein, and within the DNA binding domain 82% and 80% identical to the human and murine GCM2, respectively. The most similar GCM1 protein was murine Gcm1 which was 36% identical (full-length) and 75% identical in the DNA binding domain, confirming phylogenetic analyses which indicated that the zebrafish *gcm* gene was a member of the GCM2 subfamily (Fig. 1a). Databases contained partial sequences for *gcm2* orthologues in two other teleosts, *Takifugu rubripes* and *Tetraodon nigroviridis* and two putative *gcm1* orthologues in *Xenopus laevis*, although we found no *gcm1* orthologues in zebrafish (approximately 95% of the genome sequenced) or any fish species represented in public databases (Figs. 1a, b).

The genomic structure of the gene was strikingly similar to that of mammals, containing four introns at the start of the gene (Fig. 1c). A genomic Southern blot, using a probe corresponding to the conserved DNA binding domain revealed single fragments from several restriction enzyme digestions of genomic DNA, indicating the presence of a single copy of the *gcm2* gene in the zebrafish genome (Fig. 1d).

### Zebrafish gcm2 is expressed in the pharyngeal ectodermal epithelium and gill filament buds

We examined the expression of gcm2 during embryonic development using whole-mount in situ hybridisation and reverse transcriptase (RT) PCR (Fig. 2). Like murine Gcm2, zebrafish gcm2 expression was detected in the pharyngeal epithelium, but extended over arches 3–6 (the branchial arches) and was localised in the external (lateral), ectodermal portion of the epithelium (Figs. 2a–e). Pharyngeal expression was first detected at 32 hpf with expression



Fig. 3. Specification and patterning of pharyngeal endoderm is required for ectodermal expression of gcm2. (a, b) *casanova* is required for the expression of gcm2. gcm2 transcripts were detected by in situ hybridisation at approximately 48 hpf in wild-type,  $cas^{+/?}$ , siblings (arrowhead in a) from a cross between known *casanova* heterozygotes, but staining was absent in *casanova*,  $cas^{-/-}$  mutants (b). (c, d) *foxi1* is required for the expression of gcm2. gcm2 transcripts were detected by in situ hybridisation at 72 hpf in wild type,  $foxi1^{+/?}$  siblings from a cross between known heterozygotes for *foxi1* (arrowhead in c), but staining was severely reduced or absent in  $foxi1^{-/-}$  mutants (d).

initiating progressively in the developing branchial arches in a rostral to caudal sequence from 32 to 48 hpf. By 48 hpf, expression was observed in all four arches, where it persisted to at least day 5 (Figs. 2a–c). *gcm2* expression occurred in the gill filament buds as they evaginate from the pharyngeal ectoderm from 72 to 74 hpf, these distinctive structures (shown at 96 hpf—arrowhead Fig. 2c) give rise to the fish-specific gill filaments (Kimmel et al., 1995).

Additionally, from 10 hpf, *gcm2* was expressed in a population of cells scattered over the yolk in the epidermis (Figs. 2f and g). By 20 hpf, these cells were dispersed over the entire yolk, yolk extension and tail (Fig. 2g). These cells were located in the immediately sub-epithelial compartment (Fig. 2h) and were absent in *swirl* mutants but not in maternal zygotic *one eyed pinhead, cloche* or in *casanova* mutants

(data not shown). Taken together, these observations suggest that this *gcm2*-expressing cell population comprises nonneural ectoderm-derived epidermal cells. By virtue of their dispersed location, these cells are unlikely to be involved in pharyngeal development and so are not described further in these studies. RT-PCR analysis confirmed expression of *gcm2* from as early as 10 hpf and throughout embryogenesis and in the adult testis, brain and integument (a crude skin and adipose tissue extract) (Fig. 2i).

## gcm2 expression in the pharyngeal ectoderm requires the normal specification of the pharyngeal endoderm

We examined pharyngeal gcm2 expression in the endoderm mutants casanova and foxi1. casanova mutants



Fig. 4. Validation of *gcm2* morpholino oligonucleotide activity. (a) Representation of reagents used in a two-injection rescue experiment. Two pCS2+-based constructs were built to enable synthesis of two different *gcm2* capped mRNA transcripts. The *ATGgcm2* mRNA contained the full *gcm2* coding sequence but did not include the full binding sites for any of the morpholino oligonucleotides used. The *UTRgcm2* mRNA contained 5' UTR and the full *gcm2* coding sequence and the full binding sites for the morpholino oligonucleotides MO1, MO2 and MO3. UTR is represented in red, coding sequence in blue. (b–k) Rescue of the *gcm2* overexpression phenotype occurred only in the presence of complete morpholino oligonucleotide binding sites. In this experiment, test mRNA (50 or 100 ng/ $\mu$ L) was injected at the 1–2 cell stage and morpholinos injected via a second separate needle at the 2–4 cell stage. mRNA encoding enhanced green fluorescent protein (EGFP) was co-injected (15 ng/ $\mu$ L) with test mRNA as a tracer of mRNA delivery and as a further specificity control. Injections of *ATGgcm2* and *UTRgcm2* mRNA resulted in a highly reproducible early axis-patterning defect (c, d), which was not rescued by a separate, second injection of the mis-matched control morpholino (misMO) (e) and was not observed in uninjected embryos (b). A second separate injection of MO1, MO2 or MO3 rescued the overexpression phenotype in *UTRgcm2* mRNA injected embryos (f–h) but not in *ATGgcm2* mRNA injected embryos (i–k), nor did any morpholino affect EGFP expression. Combined scores from two separate experiments are shown in the bottom right of each panel in three categories of phenotype severity: grossly elongated embryos/ mildly dysmorphic embryos/phenotypically normal embryos. Merged false color images are shown, with green indicating the presence of EGFP.

lack endoderm (Alexander and Stainier, 1999; Aoki et al., 2002; Kikuchi et al., 2001) and we examined the progeny of known heterozygotes for *gcm2* expression. *casanova* mutants lacked pharyngeal expression of *gcm2* at 48 hpf (Figs. 3a and b). Furthermore, in phenotypically identifiable *casanova* mutants at 38 hpf (during the initiation of *gcm2* expression), *gcm2* expression was absent (n = 7 mutants amongst 29 siblings from a  $cas^{+/-} \times cas^{+/-}$  cross) indicating that endoderm was required for the initiation of *gcm2* expression and not simply for its maintenance.

We also examined gcm2 expression in *foxi1* mutants, which lack normal patterning of pharyngeal endoderm (Nissen et al., 2003; Solomon et al., 2003). *foxi1* mutants lacked pharyngeal expression of gcm2 at 72 hpf (Figs. 3c and d). Expression was also absent in *foxi1* mutants at 48 hpf (data not shown). Taken together, these data show that initiation of gcm2 expression in the external ectodermal compartment requires normal specification of the pharyngeal endoderm.

#### gcm2 is required for gill filament budding

We used morpholino (MO) oligonucleotide knock-down of transcript translation (Nasevicius and Ekker, 2000) to examine gcm2 function and uncover a requirement for gcm2 in gill filament budding and craniofacial development. To first validate MO efficacy and specificity, we took advantage of the overexpression phenotype for gcm2 (Fig. 4). gcm2 overexpression by mRNA injection led to a dose-dependent induction of axis patterning defects which was associated with the ectopic induction of *chordin* from as early as 30% epiboly (data not shown). The early embryonic lethality of this phenotype precluded classic rescue of morphant phenotype experiments. Therefore, we injected gcm2 mRNA, with and without the full MO binding sites, in the presence and absence of MOs targeted to gcm2 transcripts. MOs specifically inhibited the overexpression phenotype, but only when the full MO binding sites were present in the injected gcm2 mRNA (Figs. 4f-k). Furthermore, gcm2 MOs failed to inhibit the translation of EGFP from mRNA, which was co-injected with the gcm2 mRNA. In order to prevent binding of gcm2 to MOs in vitro, these rescue experiments were performed by injecting firstly the mRNA, followed by a separate second injection of MO. Hence, gcm2 MOs were capable of specifically targeting gcm2 mRNA in vivo, but only in the presence of intact MO binding sites. Furthermore, the temporally regulated expression of a series of well-characterised genes assayed from early somitogenesis to 3 dpf (including: gata1, spi1, l-plastin, c-myb, myeloperoxidase, apolipoproteinE, runx1, intestinal fatty acid binding protein, prox1, pdx1, insulin, crestin, dlx2) was normal in MO injected embryos, indicating the specificity of the MO phenotype (data not shown).

MO-injected embryos (morphants) displayed a characteristic combination of phenotypic defects from 3 days post-fertilisation (dpf), comprising enlarged yolks, a hypoplastic gastrointestinal tract, under-developed otoliths and decreased protrusion of the jaw, prominent at 5 dpf (Figs. 5a-d). Alcian blue-stained morphants displayed specific abnormalities of variable severity in craniofacial cartilage development (Figs. 5e-j). In uninjected and standard random-sequence MO control-injected embryos, early calcification of the cleithrum was invariably detected by calcein staining (Du et al., 2001) from 4 dpf, however, this staining was absent in 4 dpf gcm2 morphants (Figs. 5k and l). Given the ectodermally restricted expression of gcm2, these skeletal phenotypes are likely non-cell autonomous. To identify cell autonomous requirements for gcm2 in pharyngeal development, we analysed gill filament buds in wild-type and gcm2 morphant embryos. DIC microscopy of uninjected 98 hpf control embryos invariably revealed distinctive budding, whilst in gcm2 morphants, gill filament budding was severely reduced or absent (Figs. 5q, t).

We exploited the phenomenon of MO oligonucleotidemediated stabilisation of target mRNA in vivo (Oates and Ho, 2002) to examine gill filament budding in *gcm2* morphants using *gcm2* as a marker. At 80 hpf, distinctive gill filament budding was observed in wild-type embryos (Fig. 5r) whereas, morphant embryos had either no budding or severely reduced budding at this developmental stage (Fig. 5u). At 98 hpf, whilst uninjected or mis-matched MO injected controls all showed expression in gill filament buds (Fig. 5s), morphants showed no budding or severely reduced budding (Fig. 5v). Furthermore, at 76 hpf, the expression of *rag1* in the developing thymus, adjacent to the gill filament buds, was normal (Figs. 5m, n), as was the early development of the thyroid, marked by *nkx2.1* at 44 hpf (Figs. 5o, p) indicating the specificity of these phenotypes.

### Hox group 3 paralogs are broadly expressed throughout the developing pharyngeal pouches preceding gcm2 expression

To better understand the role played by gcm2 during evolutionary changes in pharyngeal morphology, we investigated the control of its expression in the developing zebrafish pharyngeal arches. Murine HoxA3 is expressed in the pharyngeal endoderm and HoxB3 in the pharyngeal ectoderm (Hunt et al., 1991a,b; Manley and Capecchi, 1995). HoxA3 likely acts cell autonomously upstream of Gcm2 in the mouse (Manley and Capecchi, 1995; Su et al., 2001). In zebrafish, hoxa3 and hoxb3 expression in the pharyngeal region occurs at times that are both earlier than and concomitant with those we describe for gcm2 expression (Hadrys et al., 2004; Prince et al., 1998; Schilling et al., 2001), but it was unclear if their expression domains include the ectoderm or endoderm. Hence, to determine if pharyngeal hoxa3 and hoxb3 expression was restricted to the neural crest, or if their expression is found in ectodermal cells where they may act cell-autonomously upstream of gcm2, we compared the expression patterns of hoxa3 and

*hoxb3* with the expression of *crestin* (marking the neural crest) and dlx2 (marking the neural crest derived mesen-chyme) (Fig. 6).

Both *hoxa3* and *hoxb3* were expressed broadly throughout the pharyngeal region at 24 hpf and were not restricted to the neural crest or neural crest derived mesenchyme, indicating that their expression is located in an appropriate region to potentially exert a direct influence over *gcm2* expression (Fig. 6). To determine if *hoxa3* and *hoxb3* expression in the pharyngeal ectoderm coincided with the initiation of *gcm2* expression, we sectioned embryos at 38 hpf, which is during the time of initiation of *gcm2* expression. At 38 hpf, *gcm2* expression was typically observed in the ectoderm of the two rostral-most branchial arches (Fig. 6i, inset). At the same time, broad expression of *hoxa3* and *hoxb3* in the developing caudal branchial arches included expression in the ectoderm (Figs. 6i, j). At this timepoint, in the more mature rostral arches, *hoxa3* and *hoxb3* expression was enriched in the mesenchyme. Later in development, at 48 hpf, sectional analysis revealed that *hoxa3* and *hoxb3* expression was predominantly restricted to the mesenchyme (data not shown). Hence, both Hox genes were expressed in the ectoderm during the period of initiation of *gcm2* expression but became more restricted to mesenchyme thereafter.

#### Hox group 3 paralogs are required for gcm2 expression

To determine whether gcm2 expression was dependent on the function of *hoxa3* and *hoxb3*, we analysed gcm2



expression in morphants for *hoxa3*, *hoxb3* and *hoxa3/hoxb3* together at 50–52 hpf and 76 hpf. Activity of the Hox group 3 MOs which were targeted to the start codon (*hoxa3*MO and *hoxb3*MO) was first validated using an inhibition of in vitro translation assay (Fig. 7i). The specificity of *hoxa3*MO and *hoxb3*MO effects was further verified by replication of observations with splice donor site targeting MOs (*hoxa3*-MO<sup>Sp</sup> and *hoxb3*MO<sup>Sp</sup>) (Table 1).

In contrast to HoxA3-deficient mice, hoxa3 morphants were wild type in appearance and displayed normal gcm2expression (Fig. 7b, Table 1). However, at 50–52 hpf, knock-down of hoxb3 led to a marked reduction in gcm2expression, with expression most commonly observed in the ectoderm of only two branchial arches (Fig. 7c, Table 1), whereas in wild-type controls, gcm2 was expressed in all four branchial arches. gcm2 expression in hoxb3morphants at 76 hpf remained reduced, but to a lesser extent (Table 1); this increase in gcm2 expression likely reflected dilution or degradation of the hoxb3MO reagent by these later stages of development.

In compound *hoxa3/hoxb3* morphants, the loss of *gcm2* expression was enhanced. At 50–52 hpf, *gcm2* expression was generally reduced to low level expression in one arch (Fig. 7d, Table 1) and although *gcm2* expression had again recovered partially by 76 hpf, it remained reduced compared to *hoxb3* morphants (Table 1). The loss of *gcm2* expression was specific to the knockdown of Hox group 3 paralog function and not due to general developmental delay because morphants showed no obvious specific morphological defects and expressed *rag1* normally at 78 hpf by single riboprobe in situ hybridisation (data not shown). Furthermore, when analysed by double in situ hybridisation for both *gcm2* and

rag1, hoxa3/hoxb3 morphants that displayed decreased gcm2 expression and decreased gill filament budding at 78 hpf retained normal expression of rag1 in the developing thymus (Figs. 7e and f).

To determine whether Hox group 3 paralogs were required for the initiation of gcm2 expression or were simply required for its maintenance, we looked at gcm2expression in hoxa3/hoxb3 morphants at 38 hpf, during the time of initiation of gcm2 expression. In both hoxa3MO/hoxb3MO and  $hoxa3MO^{Sp}/hoxb3MO^{Sp}$  double morphants, gcm2 expression was vastly reduced or absent (n = 44/47 hoxa3MO/hoxb3MO double morphants and n = 42/48  $hoxa3MO^{Sp}/hoxb3MO^{Sp}$  double morphants, expression was normal in n = 62/62 random sequence control MO-injected embryos).

To further investigate the compensation of *hoxa3* in the absence of *hoxb3*, we examined *hoxa3* expression in *hoxb3* morphants. We found that *hoxa3* staining intensity was consistently increased in *hoxb3* morphants (Figs. 7g and h), showing that *hoxa3* is up-regulated in the absence of *hoxb3* function.

#### Discussion

#### *Gill filament budding employs similar molecular regulators to parathyroid gland development*

We have shown that unlike murine Gcm2, which is expressed in third pharyngeal pouch endoderm and required for parathyroid gland development (Gunther et al., 2000), zebrafish gcm2 is expressed in the ectodermal epithelium of the branchial arches and is required for gill filament bud

Fig. 5. gcm2 is required for craniofacial development and gill filament budding. (a-d) Light microscope images of 5 dpf embryos injected with the standard control morpholino (a, b) and MO1 (c, d). (a, b) Standard control morpholino injected embryo at 5 dpf displaying normal otolith, gastrointestinal and jaw development. Mis-matched control-injected embryos (n = 160) were indistinguishable from random-sequence morpholino or uninjected control-injected embryos. (b is enlarged image of boxed section in a). (c, d) gcm2 morphants display a failure of jaw protrusion (black arrow in c), hypoplastic intestine (white arrow in d), abnormal yolk absorption and underdeveloped otoliths (red arrow in d). The morphant phenotype was generated with an ATG targeted morpholino, MO1 (approximately 7.5 ng/embryo), at 98% frequency (n = 167). The phenotype was reproduced with an injection of two morpholino oligonucleotides combined (78% frequency, n = 84): a 5' untranslated region targeted morpholino, MO2; and a second ATG targeted morpholino, MO3 (approximately 3.75 + 3.75 ng/embryo), which partially overlaps with the MO1 sequence. d is enlarged image of boxed section in c. (e-j) Morphants display specific defects in craniofacial cartilage development at 4 and 5 dpf. Four dpf uninjected and random-sequence morpholino-injected control embryos (n = 12) (e) stained with Alcian blue displayed wild-type craniofacial cartilages for this timepoint.  $gcm^2$  morphants (n = 67) displayed either "mild" defects in craniofacial cartilage development (29% of embryos scored) (f) or "severe" defects in craniofacial development (18% of embryos scored) (g). The mis-matched control morpholino failed to produce the phenotypes described (n = 27). Five dpf uninjected and random-sequence morpholino (n = 21) control embryos (h) stained with Alcian blue displayed wild-type craniofacial cartilages for this timepoint. gcm2 morphants (n = 79) displayed either "mild" defects in craniofacial cartilage development (31% of embryos scored) (i) or "severe" defects in craniofacial development (10% of embryos scored) (j). The mis-matched control morpholino failed to produce the phenotypes described (n = 35). (k, l) Reduced calcein staining in morphants. Calcein staining of the cleithrum at 4 dpf was invariable in uninjected controls (arrowhead in k) and in mis-matched morpholino injected controls (n = 43), but was absent in MO2 + MO3 injected morphants at 4 dpf (80% of embryos scored, n = 45) (l). (m, n) Normal rag1 expression in morphants. In situ hybridisation for rag1 at 76 hpf stained the developing thymus in uninjected controls (arrowhead in m) and in morphants (arrowhead in n). (o, p) Normal nkx2.1 expression in morphants. In situ hybridisation for nkx2.1 stained the developing thyroid at 44 hpf in uninjected controls (arrowhead in o) and in morphants (arrowhead in p). (q, t) Gill filament buds were absent or vastly reduced in morphants at 98 hpf. Gill filament buds were observed under DIC microscopy in uninjected controls at 98 hpf (n = 29) (q) but were absent or vastly reduced in morphants (n = 20/26 embryos for MO1 injected and n = 28/31 for MO2 + MO3 injected) (t). Buds are indicated by arrowheads, arches 3–6 are labelled, h = heart. (r, s, u, v) Gill filament buds expressing gcm2 were absent in gcm2 morphants. In situ hybridisation for gcm2 transcripts at 80 hpf in uninjected controls (r) showed the budding of gill filament buds as ruffled staining of arches 3-6 (arrowhead) (n = 20) which was absent or vastly reduced in morphants (n = 12/14 for MO1 injected, n = 33/40 for MO2 + MO3 injected) (u). In situ hybridisation for gcm2 transcripts at 98 hpf in uninjected controls (s) also showed the budding of gill filament buds (arrowhead) (n = 30) which was absent or vastly reduced in morphants (n = 16/26 MO1 injected, n = 48/50MO2 + MO3 injected) (v). Five mismatched control injected embryos were indistinguishable from uninjected control embryos at 98 hpf (n = 22).



Fig. 6. Pharyngeal *hoxa3* and *hoxb3* expression is not restricted to the neural crest and includes external cells of the ectoderm. (a–h) Flat-mounted embryos and transverse sections of embryos post in situ hybridisation at 24 hpf for *hoxa3*, *hoxb3*, *crestin* and *dlx2*. Expression of *hoxa3* (a) and *hoxb3* (c) in the pharyngeal region is detected broadly throughout all of the branchial region in flat-mounted embryos. Comparison with *crestin* expression (e) in neural crest and *dlx2* expression (g) in neural crest-derived mesenchyme reveals that *hoxa3* and *hoxb3* are not restricted to neural crest cells or derivatives. Transverse sections through embryos post in situ hybridisation for *hoxa3* (b) and *hoxb3* (d) at the level of the otic vesicle, also demonstrated that *hoxa3* and *hoxb3* were expressed not only in the neural crest and derivatives as marked with *crestin* (f) and *dlx2* (h) expression, but also in the surrounding cells, which included external, ectodermal cells. (i, j) Sectional analysis of *hoxa3* and *hoxb3* expression. Inset in (i) is a whole mount in situ hybridisation mount for *gcm2* expression at 38 hpf, showing expression in pharyngeal arches 3 and 4 but not yet in 5 and 6, indicating that this timepoint is during the initiation of *gcm2* expression (the black line indicates the approximate location of sections in i and j). ov indicates the position of the otic vesicle; brackets indicate the rostro-caudal extent of branchial staining in flat-mount preparations; and hashed brackets indicate the lateral extent of branchial staining in transverse sections. Sections are oriented with lateral to the left and medial to the right. Scale bars in b, d, f and h indicate 20 µm and in i and j indicate 10 µm.

development. *gcm2* is also required non-cell autonomously for normal pharyngeal cartilage development. As *gcm2* functions as a transcription factor, this requirement implies that inductive or permissive signalling from the ectoderm may be required in the development of other pharyngeal tissues. Interestingly, the specification of the adjacent pharyngeal endoderm is necessary for the normal initiation of gcm2 expression in pharyngeal ectoderm, indicating a requirement for inductive signalling from the endoderm for expression of this particular ectodermal transcription factor.

Somewhat unexpectedly, our observations present molecular evidence for a developmental relationship between





Fig. 7. hoxb3, and in its absence hoxa3, are required for normal gcm2 expression. (a-d) Hox group 3 paralogs are required for normal gcm2 expression. Expression of gcm2 in uninjected control embryos at 52 hpf was detected by in situ hybridisation in arches 3–6 (a) and was unchanged in hoxa3 morpholinoinjected embryos (b), but was reduced in hoxb3 morpholino-injected embryos (c) and further reduced in hoxa3 + hoxb3 morpholino-injected embryos (d). (e, f) Expression of gcm2 was reduced in hoxa3MO/hoxb3MO morphants in the presence of normal rag1 expression. Expression of gcm2 in distinctive gill filament buds and rag1 in the thymus was detected at 78 hpf in uninjected control embryos (n = 31/35 embryos) (arrowhead in e indicates rag1 expression, lower panel is an enlarged image of boxed section in e, arrowheads in lower panel indicate gill filament buds). Expression of rag1 was also detected in hoxa3MO/ hoxb3MO morphants even in the presence of reduced gcm2 expression and reduced gill filament budding (n = 41/71 with reduced gcm2, reduced budding, rag1 positive; n = 14/71 wild type; n = 16/71 with reduced gcm2, reduced budding, rag1 negative) indicating the specificity of the reduced gcm2 phenotype (arrowhead in f indicates rag1 expression, lower panel is an enlarged image of boxed section in f, arrowheads in lower panel indicate gill filament buds). (g, h) Expression of hoxa3 was increased in the absence of hoxb3. Normal expression of hoxa3 was detected by in situ hybridisation in concurrently stained 50 hpf uninjected control embryos (g) and increased intensity of staining was detected in 50 hpf hoxb3MO-injected embryos (n = 34/43) (h). To control against variation in staining, we performed in situ hybridisation on mixed control and MO injected batches, ensuring consistent staining between MO and control injected embryos. We used tail-clipping of experimental groups to provide simple identification of control or MO injected embryos post in situ hybridisation. The identical experiment using random sequence control MO embryos generated n = 33/46 hoxb3MO-injected embryos displaying more intense staining than observed in n = 48 random sequence control MO injected embryos, which was reproduced with  $hoxB3MO^{Sp}$  (data not shown). (i) Specific activity of hoxa3MO and hoxb3MO, start codon targeted morpholinos assessed by inhibition of in vitro translation assay. hoxb1a mRNA was translated efficiently in the presence of hoxa3 and hoxb3 MOs (lanes 1 and 5) and in the absence of MOs (lane 4). Translation efficiency of hoxb3 mRNA in the presence of hoxb3 MO (lane 2) represents <15% the translation product seen with hoxb3 mRNA only (lane 3). Translation efficiency of hoxa3 mRNA in the presence of hoxa3MO (lane 6) represents <5% the translation product seen with *hoxa3* mRNA only (lane 7).

mammalian parathyroids and piscine gill filaments. We show that *gcm2* is expressed in zebrafish gill filament buds and is essential for their budding morphogenesis. A requirement for *hoxa3* and *hoxb3* for the initiation of *gcm2* expression parallels the relationship between murine *HoxA3* and *Gcm2* (Manley and Capecchi, 1995; Su et al., 2001). Interestingly, mutations in another gene expressed in the parathyroid glands, *Gata3*, also led to familial hypoparathyroidism in humans identifying another important regulator of parathyroid development in mammals (Debacker et al., 1999; George et al., 1994; Nesbit et al., 2004). Like *gcm2*, the zebrafish *gata3* ortholog is also expressed throughout the gill filament buds (Trede et al., 2001). Hence, two known molecular regulators of mammalian parathyroid development have restricted expression in the zebrafish gill filament buds.

Despite this striking molecular similarity, the anatomy of gill filament morphogenesis is divergent from the anatomy of parathyroid development. The gills are derived from pharyngeal arches 3–6 and bud from the external ectodermal epithelium (Goodrich, 1930; Hyman, 1942), whilst the parathyroids are derived from the third pharyngeal pouch endoderm in mice (pouches 3 and 4 in humans) at the immediate endoderm–ectoderm epithelial junction (Gordon et al., 2001).

Although we have observed significant similarity between the molecular determination of gill filaments and

Table 1Extent of gcm2 expression in Hox group 3 paralog morphants

	Severity of gcm2 loss <sup>a</sup> (%)						
	Normal	1+	2+	3+	4+	5+	п
Embryos scored a	at 52 hpf						
Control	100	0	0	0	0	0	55
(uninjected)							
Random	100	0	0	0	0	0	27
sequence MO							
hoxa3MO	97	0	0	0	0	3	33
hoxa3MO <sup>Sp</sup>	100	0	0	0	0	0	15
hoxb3MO	5	8	21	63	3	0	38
hoxb3MO <sup>Sp</sup>	0	0	22	35	32	11	37
hoxa3MO +	3	0	3	27	48	19	37
hoxb3MO							
hoxa3MO <sup>Sp</sup> +	0	0	5	21	39	35	82
hoxb3MO <sup>Sp</sup>							
Embryos scored a	at 76 hpf						
Control (uninjected)	100	0	0	0	0	0	40
hoxa3MO	100	0	0	0	0	0	43
hoxb3MO	45	53	0	2	0	0	55
hoxa3MO +	10	54	10	23	0	3	31
hoxb3MO							

<sup>a</sup> Scoring of severity was according to the following categories: normal = gcm2 expression was present in all (4/4) branchial arches and normal in extent. 1+ = gcm2 expression was present in all (4/4) branchial arches but reduced in extent. 2+ = gcm2 expression was present in 3/4 branchial arches. 3+ = gcm2 expression was present in 2/4 branchial arches. 4+ = gcm2 expression was present in 1/4 branchial arches. 5+ = gcm2 expression was absent in all branchial arches.

parathyroids, we do not suggest that gill filaments play the physiological role of parathyroid glands. Furthermore, we were unable to detect expression of parathyroid hormone in any pharyngeal-derived organs in zebrafish embryos and early larvae (BH, GL, paper in preparation).

#### Upstream roles for Hox group 3 paralogs

In mice, it is likely that *HoxA3* regulates *Gcm2* directly (Manley and Capecchi, 1995), although direct regulation remains to be shown at the molecular level. Murine HoxB3 is unlikely to regulate Gcm2 as it is not expressed in the pharyngeal endoderm, but is enriched in the surface ectoderm (Hunt et al., 1991a,b; Manley and Capecchi, 1995). In zebrafish, hoxa3 and hoxb3 are expressed throughout the pharyngeal region at 24 hpf, preceding gcm2 expression, and are more broadly expressed than markers of neural crest and neural crest-derived mesenchyme (Fig. 6). Sectional analysis at these timepoints, and also at 38 hpf (during the initiation of gcm2 expression) revealed broad expression of hoxa3 and hoxb3 in the developing branchial arches (Fig. 6) and included expression in the ectoderm during gcm2 initiation. Hence, Hox group 3 paralogs may act cell autonomously in the ectoderm to directly regulate gcm2 expression.

Injection of *hoxb3* MO but not *hoxa3* MO led to a loss of pharyngeal expression of *gcm2* which was further reduced

when both MOs were injected together. Hence, in zebrafish, it is hoxb3 that is most critical for gcm2 expression; only in the absence of hoxb3 is a role for hoxa3 uncovered, a strikingly different scenario to that observed in the mouse. At this point, it is unclear whether this requirement constitutes direct transcriptional control, although the timing of expression of Hox group 3 paralogs and gcm2 in the ectoderm and the failure of initiation of very early gcm2expression in Hox group 3 morphants suggest that this is possible.

The altered expression of gcm2 we observe may also be due to an altered identity of the branchial arches in the absence of Hox group 3 paralogs. The loss of Hox group 3 paralogs in the branchial arches may lead to a homeotic transformation with loss of segmental identity. It is possible that Hox group 2, 4 or 5 paralogs may exert their influence in the absence of *hoxa3* and *hoxb3*. However, this notion is not supported by a previous detailed analysis of arch identity using the start codon targeting MO reagents reported here, which failed to reveal any such transformations of identity (Hunter et al., unpublished).

#### Evolutionary implications

The expanded expression of  $gcm^2$  in zebrafish, relative to mammals, leads us to speculate that the ancestral state of  $gcm^2$  expression may have resembled the domain seen in zebrafish. Although  $gcm^2$  expression has not been characterised in a third modern vertebrate taxon, previous studies of the fossil record have supplied a third point of evidence. In the Devonian period, the most ancient known tetrapods *Acanthostega* and *Ichthyostega* bore deeply grooved gill bars consistent with fish-like breathing and the presence of gill filaments (Clack et al., 2003; Coates and Clack, 1991). As we demonstrate that gill filament formation requires  $gcm^2$  activity, and hence may be considered a phenotypic proxy for  $gcm^2$  expression, fish-like breathing in these ancient tetrapods implies an expanded role for  $gcm^2$  in these animals in comparison with modern tetrapods.

We speculate that gcm2 was likely broadly expressed in the gill arches, but that its expression was rapidly restricted during the transition from marine to terrestrial vertebrates and appeared for the first time in pharyngeal endoderm, in the parathyroid primordia. Consistent with this hypothesis, the number of parathyroid glands (and presumably gcm2expressing primordia) varies remarkably throughout the evolution of terrestrial vertebrates, with mice having one pair of parathyroids, humans two pairs (derived from the dorsal region of the arches in mammals) and some reptiles having three pairs (derived from the ventral region of the arches) (Hoar and Randall, 1969; Hyman, 1942). Testing of this hypothesis requires further analysis of gcm2 expression in evolutionarily divergent extant vertebrates such as sharks, skates and lungfish, which may help to delineate how these organ primordia changed throughout evolution.

Addendum. While this manuscript was under review, Hanaoka et al., (Mechanisms of Development (2004) 121 (10) 1235-47) reported that gcmb (gcm2) was required for pharyngeal cartilage development, corroborating our data describing the non-cell autonomous role of gcm2. In the same manuscript, the injection of a morpholino targeting fgf3 led to a loss of gcm2 expression in the pharyngeal ectoderm, suggesting that fgf3 is likely the signal from the endoderm required for gcm2 expression in the pharyngeal ectoderm, as hypothesised in this manuscript.

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