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A temperature-sensitive mutation in the *nodal*-related gene *cyclops* reveals that the floor plate is induced during gastrulation in zebrafish

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SUMMARY

The floor plate, a specialized group of cells in the ventral midline of the neural tube of vertebrates, plays crucial roles in patterning the central nervous system. Recent work from zebrafish, chick, chick-quail chimeras and mice to investigate the development of the floor plate have led to several models of floor-plate induction. One model suggests that the floor plate is formed by inductive signalling from the notochord to the overlying neural tube. The induction is thought to be mediated by notochord-derived Sonic hedgehog (Shh), a secreted protein, and requires direct cellular contact between the notochord and the neural tube. Another model proposes a role for the organizer in generating midline precursor cells that produce floor plate cells independent of notochord specification, and proposes that floor plate specification occurs early, during gastrulation.

We describe a temperature-sensitive mutation that affects the zebrafish Nodal-related secreted signalling factor, Cyclops, and use it to address the issue of when the floor plate is induced in zebrafish. Zebrafish *cyclops* regulates the expression of *shh* in the ventral neural tube. Although null mutations in *cyclops* result in the lack of the medial floor plate, embryos homozygous for the temperature-sensitive mutation have floor plate cells at the permissive temperature and lack floor plate cells at the restrictive temperature. We use this mutant allele in temperature shift-up and shift-down experiments to answer a central question pertaining to the timing of vertebrate floor plate induction. Abrogation of Cyc/Nodal signalling in the temperature-sensitive mutant embryos at various stages indicates that the floor plate in zebrafish is induced early in development, during gastrulation. In addition, continuous Cyclops signalling is required through gastrulation for a complete ventral neural tube throughout the length of the neuraxis. Finally, by modulation of Nodal signalling levels in mutants and in ectopic overexpression experiments, we show that, similar to the requirements for prechordal plate mesendoderm fates, uninterrupted and high levels of Cyclops signalling are required for induction and specification of a complete ventral neural tube.

Key words: Floor plate, Nodal signalling, *cyclops*, Zebrafish, Shh, Twhh, Organizer, Temperature-sensitive mutation, Gastrulation, TGFβ

INTRODUCTION

The floor plate is a specialized group of cells in the ventral neural tube of vertebrates and plays a crucial role in patterning the central nervous system. The specification of motoneurones, interneurones and differentiation of oligodendrocytes has been shown to require a functional floor plate, which secretes the glycoprotein, Sonic hedgehog (Shh) (Ericson et al., 1996; Orentas and Miller, 1996; Poncet et al., 1996; Pringle et al., 1996; Briscoe et al., 2001; Lewis and Eisen, 2001). The floor plate also provides guidance cues that are essential for the axonal outgrowth of many neurones (Colamarino and Tessier-Levigne, 1995; Matise et al., 1999).

Studies in several vertebrates (Placzek et al., 1990; Lawson and Pedersen, 1992; Le Douarin et al., 1998) that investigated the development of the floor plate led to several models for its induction. One model proposes that a signalling cascade mediated by the Shh protein secreted from the notochord induces the floor plate in the overlying neural tube (Placzek et al., 2000), and mutations in the mouse *Shh* gene indeed result in floor plate deficiencies (Chiang et al., 1996). Moreover, grafting experiments in the chick have indicated that floor-plate markers can be induced in ectopic locations of the neural tube by signals from the notochord, or the floor plate itself, or by the expression of SHH protein in ectopic locations of the neural tube (van Straaten et al., 1985; van Straaten et al., 1988; Placzek et al., 1990; Placzek et al., 1991; Yamada et al., 1991; Marti et al., 1995; Roelink et al., 1995; Ericson et al., 1996).

However, recent experiments in the chick, as well as analyses of zebrafish mutants suggest that the floor plate may

be induced independent of notochord specification (Halpern et al., 1997; Le Douarin et al., 1998; Le Douarin and Halpern, 2000; Charrier et al., 2002). For example, zygotic mutations in zebrafish *cyclops* (*cyc*), which encodes a Nodal-related secreted signalling factor (Hatta et al., 1991; Rebagliati et al., 1998; Sampath et al., 1998), and *one eyed pinhead* (*oep*), which encodes an essential co-factor for Nodal signalling (Gritsman et al., 1999), result in the lack of a floor plate, in spite of the presence of a morphologically normal notochord expressing *shh* (Strahle et al., 1997; Schier et al., 1997). On the other hand, mutations in the *flh* and *ntl* genes, which are required for the formation of the notochord, result in embryos that exhibit a patchy or wider floor plate, respectively (Halpern et al., 1997). Furthermore, medial floor plate cells are not abolished by mutations in the zebrafish *shh* gene (Schauerte et al., 1998) or its receptor, *smoothened* (Chen et al., 2001; Varga et al., 2001), or by abrogation of Hedgehog signalling using antisense knockdown with morpholino-modified oligomers (Etheridge et al., 2001). Therefore, an alternate model proposes that the floor plate is induced in the organizer-derived midline precursor cells (Le Douarin and Halpern, 2000; Charrier et al., 2002). As the precursor cells give rise to both the notochord and the floor plate, this model predicts that floor-plate induction takes place early during gastrulation. A key unresolved issue in the models pertains to the timing of floor plate induction.

We have isolated a temperature-sensitive mutation in the zebrafish *cyc* locus. In contrast to null mutations in *cyc*, embryos homozygous for the cyc^{sg1} mutation manifest variable *cyc* phenotypes at 22°C. Mutant embryos exhibit variably fused eyes, ventral curvature and patchy to complete floor plate, with motoneurones that may or may not be at their normal positions. At 28.5°C, cyc^{sg1} mutant embryos are indistinguishable from *cyc*-null mutant embryos, with fused eyes, lack of medial floor plate cells and ventral curvature. Using this allele in temperature shift-up and shift-down experiments, we show that Cyc function is essential at gastrulation to induce the floor plate in zebrafish. By modulating Nodal signalling levels in mutants, and by overexpressing *cyc* in wild-type embryos, we show that high levels of Cyc signalling are required for induction of the floor plate. Furthermore, we show that continuous and high levels of Cyc signalling during gastrulation are essential for formation of a complete ventral neural tube. These results show that the floor plate inducing activity of Cyc is essential during gastrulation, and that it is required at multiple steps of the floor plate induction pathway for the development of a complete ventral neural tube.

MATERIALS AND METHODS

Zebrafish strains and maintenance

Adult fish were maintained and reared as described in Westerfield (Westerfield, 1994). The mutant lines used in this analysis are cyc^{m294}/+ (Schier et al., 1996), cyc^{tf219}/+ (Brand et al., 1996), cyc^{b16}/+ (Hatta et al., 1991), sqt^{cz35}/+ (Heisenberg and Nusslein-Volhard, 1997; Feldman et al., 1998) and cyc^{sg1}/+ (this work). Double mutants were generated by crossing heterozygous cyc^{m294}/+ to sqt^{cz35}/+ and cyc^{sg1}/+ to sqt^{cz35}/+. The zebrafish wild-type strain AB (Johnson et al., 1994) was used for out-crosses, and the polymorphic WIK strain was used for mapping (Rauch et al., 1997). Embryos were collected after natural matings and staged according to Kimmel et al. (Kimmel et al., 1995).

cyc allele screen, mapping and sequencing

Adult zebrafish males of the AB strain were mutagenized with the chemical ethyl nitrosourea as described (Riley and Grunwald, 1995), and mosaic F1 progeny were screened for non-complementation with cyc^{tf219}/+ fish. Putative mutant fish were subsequently tested with other known *cyc* alleles as well as other mutants affecting the Nodal signalling pathway. Identified heterozygous fish were out-crossed to AB or to WIK fish. Embryos from identified heterozygous fish in the next generation were split into two groups at the one-cell stage, allowed to develop at 28.5°C or at 22°C until prim-5 stage, and analysed for *cyc* phenotypes of fused eyes, ventral curvature and the floor plate. Mapping was carried out using PCR on a AB/WIK mutant panel (n=80 haploid and 1200 diploid embryos) using primers flanking a CA repeat in the 3′-untranslated region of *cyc* (Sampath et al., 1998). For identifying the mutation, genomic DNA was isolated from single cyc^{sg1} mutant embryos at 24 hours post-fertilization (hpf) and used as templates for sequencing. In addition, DNA and RNA were extracted from individual cyc^{sg1} homozygous embryos at shield stages using TRIZOL reagent (Gibco, BRL), and single embryos were genotyped using the *cyc* CA repeat marker. RT-PCR was carried out using pooled RNA from identified cyc^{sg1}mutant embryos and the nucleotide sequence was determined.

Genotyping

The genotype of sqt^{cz35} mutant embryos and cyc^{m294} mutant embryos was determined as described (Feldman et al., 1998; Sampath et al., 1998). For determining the genotype of cyc^{sg1} homozygous mutant embryos, genomic DNA was isolated from single embryos (live or after analysis of in situ hybridization patterns), and PCR was performed with the primers 5′-AACAGGAGCTACCGAGCAGGC-3′ and 5′-ACTGGCCCCGTCCTGCTGCT-3′. The PCR products were digested with the restriction enzyme *Pvu*II (New England Biolabs), and analysed by agarose gel electrophoresis.

Temperature shift experiments

Embryos obtained from matings of cyc^{sg1}/+ fish were split into two groups at the one-cell stage, and raised at 22°C and 28.5°C, respectively. At regular intervals from 50% epiboly to 10-somite stages, embryos at 22°C were shifted to 28.5°C. Conversely, embryos raised at 28.5°C were shifted to 22°C at the same intervals. For temperature pulse experiments, embryos were incubated at either 22°C or 28.5°C with a brief shift-up or shift-down period during mid-gastrulation. Embryos were fixed at 100% epiboly or prim-5 stages for in situ hybridization with various markers.

Generation of constructs

The cyc^{sg1} mutation was introduced into pCS2cyc^{+} (Sampath et al., 1998) by PCR-based mutagenesis. The Flag epitope-tagged pCS2cyc^{+FLAG} and pCS2$cyc^{sg1FLAG}$ constructs were generated by PCR-based methods and their nucleotide sequence was confirmed. In both constructs, the Flag epitope was fused in frame after the cleavage site, between Val 385 and Arg 386 in Cyc.

Embryo injections and animal cap assays

The plasmids pCS2cyc^{+}, pCS2cyc^{+FLAG}, pCS2cyc^{m294}, pCS2cyc^{sg1}, and pCS2$cyc^{sg1FLAG}$ were linearized with *Not*I, and sense strand capped mRNA was synthesized with SP6 RNA polymerase using the mMESSAGE mMACHINE system (Ambion). In vitro synthesised RNA was injected into one- to four-cell stage wild-type embryos. Animal caps were dissected at late blastula stages and cultured as described (Sagerstrom et al., 1996; Dheen et al., 1999) until sibling stage 80% epiboly at 22°C or 28.5°C. Animal cap explants and embryos at various stages were fixed for antibody staining or in situ hybridization with various markers.

Cell culture

Cos-7 cells were cultured at 37°C in DMEM (Gibco-BRL) containing

10% foetal bovine serum (Sigma), 10 U/ml penicillin and 10 mg/ml streptomycin sulphate (Sigma). The plamids pCS2cyc^{+FLAG} and pCS2$cyc^{sg1FLAG}$ were transfected into Cos-7 cells using the Superfect transfection reagent (Qiagen). Cells were fixed after 24 hours in 4% paraformaldehyde and processed for detection of the Flag epitope.

In situ hybridization

Whole-mount in situ hybridization was performed as described (Sampath et al., 1998) on embryos fixed at 100% epiboly (10 hpf at 28.5°C or 20 hpf at 22°C), six-somite (12 hpf at 28.5°C or 24 hpf at 22°C) or prim-5 stages (24 hpf at 28.5°C or 48 hpf at 22°C). The following plasmids were linearized and antisense probes were synthesized by in vitro transcription: pBSshh (*Eco*RI, T7) (Krauss et al., 1993), pBStwhh (*Pst*I, T7) (Ekker et al., 1995), pBSislet2 (*Eco*RI, T7) (Appel et al., 1995), pBShgg1 (*Xba*I, T7) (Thisse et al., 1994), pBSgsc (*Eco*RI, T7) (Stachel et al., 1993), pBSflh (*Eco*RI, T7) (Talbot et al., 1995). Single- or double-colour in situ hybridization was performed as described (Sampath et al., 1998). For digoxigenin-labelled probes, BM purple substrate (Roche) was used; for fluorescein-labelled probes, fast red (Roche) or 4-iodonitrotetrazolium violet (Molecular Probes) were used. For cryosections, whole-mount bicolour in situ hybridized embryos were embedded in 1.5% agarose:30% sucrose blocks. The blocks were frozen and sections were obtained on a Leica CM 1900 cryomicrotome at 16 μm intervals.

Immunostaining

Embryos and animal caps were fixed in 4% paraformaldehyle at 4°C overnight. Embryos were incubated with a monoclonal antibody raised against the zn-5 epitope (Trevarrow et al., 1990), and colour was developed using the ABC kit (Pierce) with the substrate diaminobenzidine (Sigma). Animal caps and Cos-7 cells were incubated with an anti-Flag polyclonal antibody (Sigma), and detected with an anti-rabbit secondary antibody conjugated with Alexa 568 (Molecular Probes). Optical sections were obtained at 0.5 μm intervals on a Zeiss Axiovert 200M microscope, and images were processed using the Zeiss LSM image browser software.

RESULTS

Isolation of cyc^{sg1}, a temperature-sensitive mutation in the *cyc* locus

In a mutagenesis screen for new mutations in the *cyc* locus, we identified one mutant, *sg1*, which did not complement the cyc^{m294}, cyc^{tf219} and cyc^{b16} mutations (Hatta et al., 1991; Brand et al., 1996; Schier et al., 1996), and mapped to the *cyc* locus (Talbot et al., 1998; Sampath et al., 1998). In contrast to null mutations in the *cyc* locus, embryos homozygous for the cyc^{sg1} mutation exhibit variably fused eyes (Fig. 1A-E) and variable degrees of ventral curvature (Fig. 1F-J) at 22°C, the permissive temperature for cyc^{sg1} mutants. The mutation is incompletely penetrant at 22°C (Table 1), with only 2-3% of homozygous mutants exhibiting the 'classic' *cyc* phenotype (Hatta et al., 1991). In addition, only 50% (n=25) of the homozygous mutant embryos at 22°C manifest the *cyc* phenotypes (Table 1). The mutation is fully penetrant at 28.5°C (Table 1), the restrictive temperature for cyc^{sg1}mutant embryos.

Nucleotide sequence analysis revealed an A to T transversion at position 853 of the *cyc*-coding sequence, which results in a premature stop codon (Fig. 2A,C). The mutation also introduces a site for the restriction enzyme *Pvu*II in the *cyc* cDNA (Fig. 2B). To confirm if the A-T transversion causes the temperature-sensitive (ts) phenotype, synthetic mRNA encoding Cyc^{sg1} was generated and microinjected into wild-type embryos. Microinjection of in vitro synthesized cyc^{sg1} mutant mRNA into wild-type embryos resulted in *cyc* overexpression phenotypes (Rebagliati et al., 1998; Sampath et al., 1998) at 22°C, shown by the expansion (Table 2 and Fig. 2E) or duplication (Fig. 2F,G) of *shh*-expression domains. By contrast, at 28.5°C, similar to embryos injected with cyc^{m294} mutant RNA, embryos injected with cyc^{sg1} mRNA were indistinguishable from control embryos (Table 2 and Fig. 2D), confirming that the A-T transversion in cyc^{sg1} is responsible for the temperature-sensitive phenotype.

To detect wild-type and mutant proteins, synthetic mRNA encoding Cyc^{+FLAG} or $Cyc^{sg1FLAG}$ was injected into wild-type embryos, and animal cap explants dissected from the injected embryos were processed for detection of the Flag epitope. Animal caps loaded with either wild-type or mutant Flag-

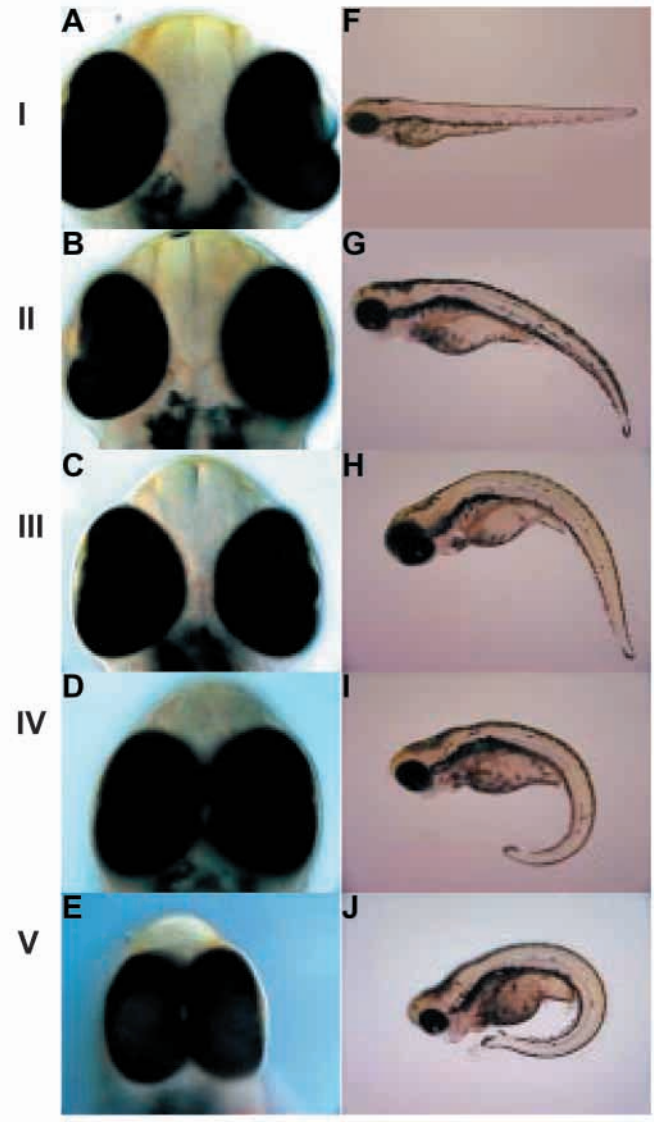

Fig. 1. Variable fusion of the eyes (A-E) and ventral curvature (F-J) in protruding mouth stage (6 days post fertilization at 22°C) cyc^{sg1} mutant embryos maintained at 22°C. Class I (A,F) represents the mildest phenotype similar to wild-type embryos, and class V (E,J) represents the most severe phenotypes, similar to null mutations in *cyc*. At 28.5°C, only class V phenotypes are observed.

Table 1. Phenotypes manifested by homozygous *cyc^{sg1}* mutant embryos at the permissive (22°C) and restrictive (28.5°C) temperatures

Phenotype		Total number of embryos	Mutant embryos at 22°C	% mutant	Total number of embryos	Mutant embryos at 28.5°C	% mutant
Fusion of eyes	I		7	3.2		0	0
	II		10	4.6		0	0
	III		9	4.1		0	0
	IV		1	0.5		0	0
	V		4	1.8		71	27.1
Ventral curvature	I		5	2.3		0	0
	II		3	1.4		0	0
	III		4	1.8		0	0
	IV		11	5.0		0	0
	V		8	3.7		71	27.1
Totals		218	31	14.2	262	71	27.1

Mutant embryos were scored for fusion of eyes and ventral curvature of the body. Variable phenotypes were observed at 22°C, whereas at 28°C, the mutants exhibited only severe *cyclops* phenotypes. Phenotypes have been classified based on increasing severity, with class I representing the mildest phenotype (similar to wild-type embryos) and class V representing severe phenotypes (similar to null mutations in *cyc*). In addition, at 28°C, the expected Mendelian segregation was observed, whereas at 22°C, only 14.2% of the embryos showed mutant phenotypes.

tagged *cyc* RNA show localization of the wild-type (not shown) and mutant protein at 22°C (Fig. 2K). By contrast, at 28.5°C, only the wild-type protein is detected (Fig. 2I), whereas Cyc^{sg1FLAG} is not detected (Fig.2L), similar to control explants (Fig. 2H). In situ hybridization with *gsc* in the animal cap explants showed expression in explants from *cyc*^{sg1} injected embryos at 22°C but not at 28.5°C (data not shown). Cos-7 cells transfected with the plasmids pCS2*cyc*$^{+FLAG}$ and pCS2*cyc*^{sg1FLAG} show localization of the Cyc^{+FLAG} protein at 37°C (Fig. 2J), but not the Cyc^{sg1FLAG} protein (Fig. 2M).

Analysis of markers of mesendoderm, ventral neural tube and motoneurones in *cyc^{sg1}* mutant embryos

Expression of *cyc* transcripts in gastrula stage *cyc^{sg1}* mutant embryos at 22°C is similar to that seen in wild type embryos (Fig. 3A,B) or reduced (Fig. 3D,E). At 28.5°C, similar to ENU-induced null mutations in the *cyc* locus (Rebagliati et al., 1998; Sampath et al., 1998), *cyc* transcripts in *cyc^{sg1}* mutants are reduced by mid-gastrula stages (Fig. 3C), and are not detected by the end of gastrulation (Fig. 3F). Analysis of expression of *goosecoid* (*gsc*), a marker of the prechordal plate mesendoderm (Thisse et al., 1994), which is reduced in *cyc* null mutants, reveals variably reduced (Fig. 3I,J) to normal (Fig. 3G) prechordal plate mesendoderm in *cyc^{sg1}* mutants at 22°C, compared with those at 28.5°C (Fig. 3H).

Table 2. Overexpression of cyc^{sg1} mRNA results in duplication or expansion of the axis at 22°C but not at 28°C

Injected mRNA (5 pg)	Temperature	Total (*n*)	Number of embryos with expansion or duplication of *shh* expression domain (%)
cyc^{+}	22°C	138	100 (72.5)
	28°C	120	85 (70.8)
cyc^{m294}	22°C	115	0 (0.0)
	28°C	134	0 (0.0)
cyc^{sg1}	22°C	150	85 (56.7)
	28°C	133	0 (0.0)

In situ hybridization with the early marker of the floor plate, *tiggy-winkle hedgehog* (*twhh*), shows patterns that are either comparable with wild-type embryos or reduced in *cyc^{sg1}* mutants at 22°C (Fig. 4A-C). At 28.5°C, similar to null mutations in *cyc*, *twhh* expression is not detected in the midline (Fig. 4D) of *cyc^{sg1}* mutants. Strikingly, expression of *shh* in prim-5 stage *cyc^{sg1}* mutants reveals a range of patchy to complete floor plates at 22°C (Fig. 4E-G), whereas at 28.5°C, similar to null mutations in *cyc* (Hatta et al., 1991), there is a complete lack of medial floor plate cells (Fig. 4H). The *cyc^{sg1}* embryos with patchy *shh* expression reveal the intermittent presence of floor plate cells throughout the length of the embryo (Fig. 4E), with gaps in the anterior of the embryo but a fairly complete floor plate in the trunk (Fig. 4F), or with normal *shh* expression in the anterior and gaps in the trunk (Fig. 4G).

Primary motoneurones revealed by *islet2* expression also show a range of phenotypes in *cyc^{sg1}* mutants at 22°C (Fig. 5B-D) compared with wild-type siblings (Fig. 5A). The position of primary motoneurones may be similar to that seen in wild-type embryos, either with a *shh*-positive medial floor plate (Fig. 5B), or even in the absence of a *shh*-positive floor plate (Fig. 5C). Alternatively, in the absence of a medial floor plate, similar to null mutations in *cyc* (Beattie et al., 1997), the primary motoneurones collapse in the midline (Fig. 5D). Immunostaining using antibodies raised against the zn5 epitope to detect retinal ganglion cell axons in the anterior of the embryo (Fig. 5E-G), and secondary motoneurones in the trunk (Fig. 5H-J) show patterns comparable with wild-type embryos (Fig. 5E,F,H,I) in *cyc^{sg1}* mutants raised at 22°C, in contrast to those raised at 28.5°C (Fig. 5G,J).

Cyclops function is essential at mid-gastrula stages for induction of the floor plate

A fundamental question regarding the induction of the floor plate is when this event takes place (Dodd et al., 1998; Le Douarin and Halpern, 2000; Placzek et al., 2000). Because embryos homozygous for the temperature-sensitive mutation, *cyc^{sg1}*, have medial floor plate cells at the permissive temperature and lack them at the restrictive temperature, we

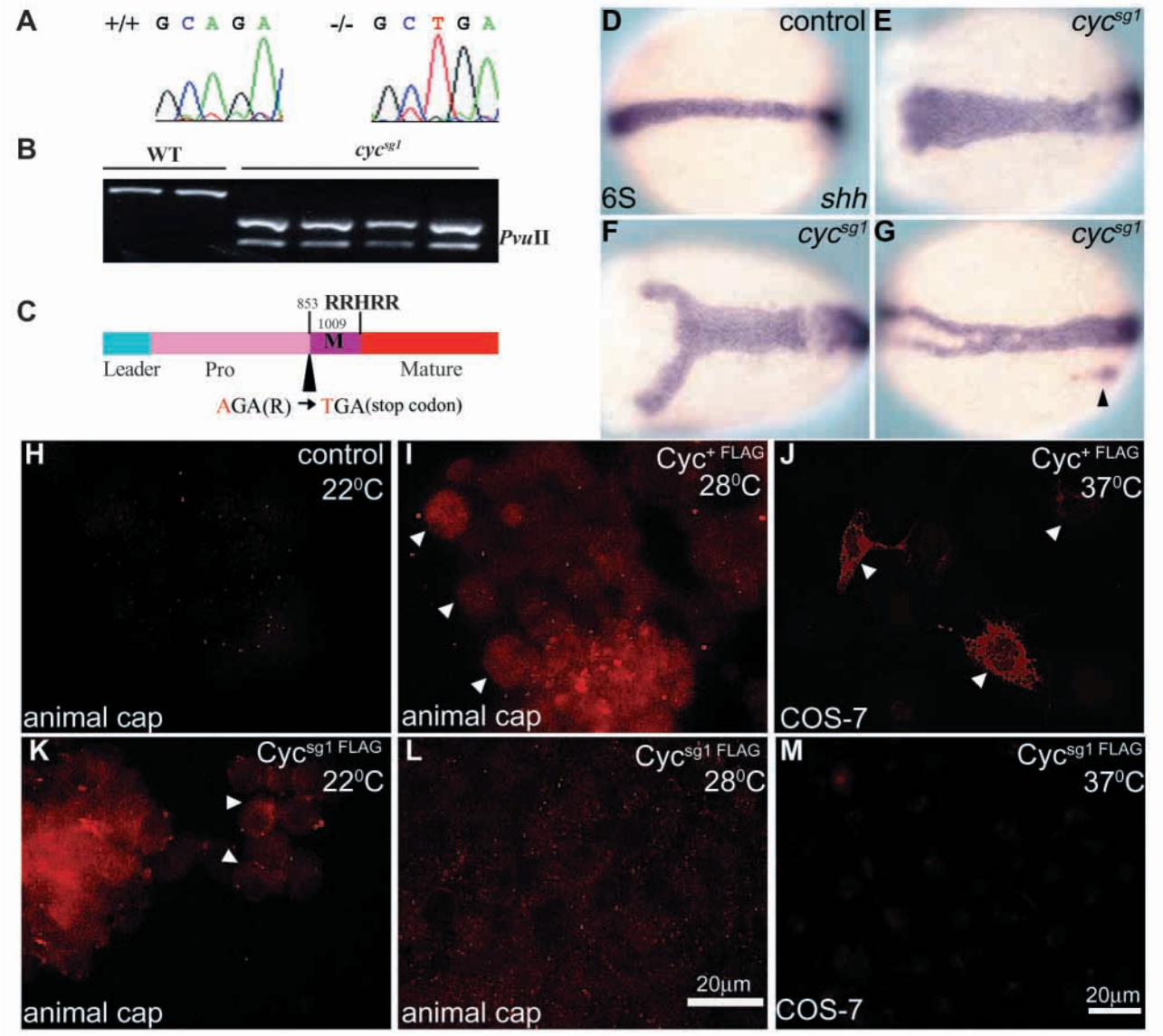

Fig. 2. Identification of the molecular lesion in *cyc*^{sg1}. (A) Nucleotide sequence electropherogram showing an A to T transversion at position 853 of the coding sequence of *cyc*. (B) The mutation introduces a new site for the restriction enzyme *Pvu*II, seen in digests of DNA amplified from individual *cyc*^{sg1} mutant embryos, when compared with wild-type embryos. (C) Schematic representation of the leader, pro and mature ligand domains of Cyc, with the cleavage site and Arg-Stop change indicated (black arrowhead). Expression of *shh* in control embryos (D) compared with expanded (E) or multiple (F,G) domains in embryos injected with *cyc*^{sg1} mutant RNA (E-G) and incubated at 22°C. Arrowhead in G indicates an additional axis in the posterior. (D-G) Dorsal views. (H-L) Expression of wild-type Cyc^{+FLAG} protein (I) compared with Cyc^{sg1FLAG} mutant protein (K,L) in animal cap explants incubated at 22°C (K) or 28.5°C (L), and control explants (H). Cos-7 cells transfected with pCS2*cyc*$^{+FLAG}$ (J) or pCS2*cyc*^{sg1FLAG} (M) show localization of Cyc^{+FLAG} protein (J) but not of Cyc^{sg1FLAG}(M). The weak nuclear staining in pCS2*cyc*^{sg1FLAG} transfected cells was detected in untransfected controls as well (not shown). White arrowheads indicate cells expressing high levels of protein. Scale bars: in L, 20 µm for H,I,K,L; in M, 20 µm for J,M.

used the *cyc*^{sg1} allele to address this question. Embryos collected from matings of *cyc*^{sg1}/+ heterozygous fish were incubated at 22°C and shifted to 28.5°C (22-28 shift) to abrogate Cyclops function at various stages of gastrulation and segmentation. In these temperature shifts, the floor plate should develop until the point in embryogenesis when Cyclops is essential for this event. Interestingly, *cyc*^{sg1} mutant embryos shifted to 28.5°C at early gastrula stages did not show any medial floor plate cells (n=42) as assessed by expression of the early markers of the floor plate, *twhh* and *shh* (Fig. 6A), as well as the markers of differentiated floor plate cells, *f-spondin2* (*spon1b* – Zebrafish Information Network) and *col2a1* (data not shown). Furthermore, 22-28 shifts performed after midgastrula stages (75% to 80% epiboly) resulted in the presence of medial floor plate cells in ~75% of mutant embryos (n=110; genotype confirmed by PCR) (Fig. 6A,C,D).

Conversely, embryos were incubated at 28.5°C, and shifted down to 22°C (28-22 shift) at various stages to determine if medial floor plate cells could be rescued in these embryos. When 28-22 shifts were performed at early to mid-gastrula

stages, more than 80% of mutant embryos exhibited medial floor plate cells (*n*=94; genotype confirmed by PCR) (Fig. 6B). The proportion of mutant embryos with *shh*-positive floor-plate cells decreases as shifts were performed later in gastrulation (Fig. 6B). A 28-22 shift-down after 80% epiboly failed to induce any medial floor plate cells (*n*=188), similar to embryos incubated at 28.5°C alone until 24 hpf (*n*=93). These results indicate that Cyclops is required for inducing the floor plate between 70 and 80% epiboly.

Interestingly, we found that embryos that were shifted down to the permissive temperature at 50% or 60% epiboly showed a complete floor plate and ventral neural tube, throughout the entire length of the neuraxis (Fig. 6E), whereas shift-down at later stages resulted in rescue of patches of floor-plate cells (Fig. 6F). The patches were distributed throughout the length of the neuraxis, regardless of the stage at which the embryos were shifted. In addition, embryos from 28-22 shift-down experiments at mid-gastrula stages typically showed longer stretches of cells expressing floor plate markers than embryos from 22-28 shifts (Fig. 6C,F). These results suggest that Cyclops function may be required first for induction of the floor plate from its precursors early during gastrulation, and, subsequently, for the complete development of the floor plate along the entire length of the neuraxis.

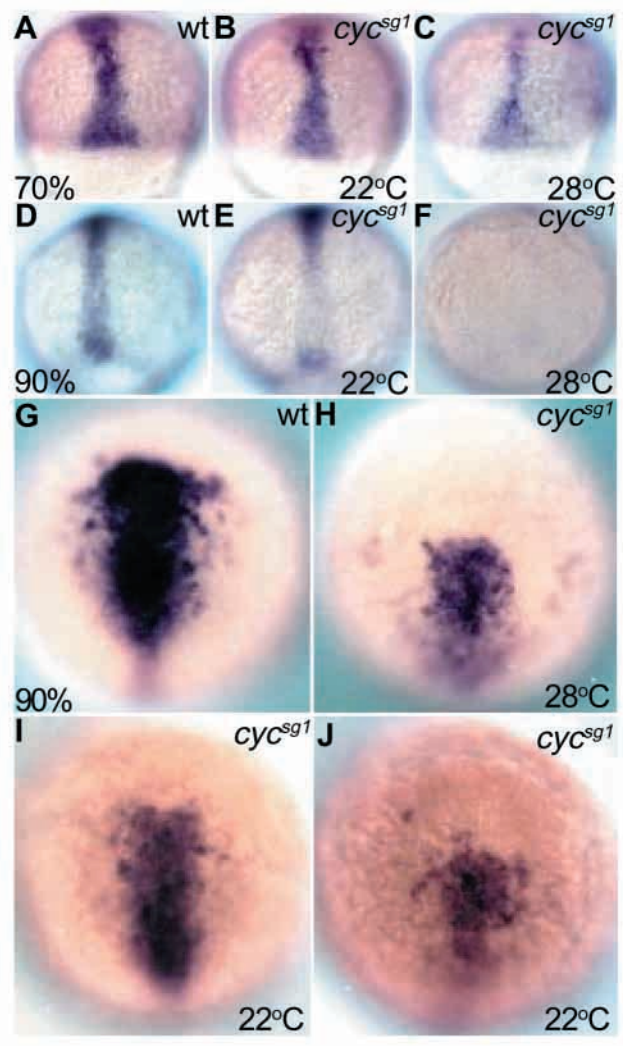

Fig. 3. Expression of *cyc* and *gsc* transcripts in *cyc*^{sg1} mutant embryos. (A-F) Dorsal views; (G-J) anterior views. At 70% epiboly (A-C) as well as 90% epiboly (D-F), *cyc* transcripts in *cyc*^{sg1} mutants (B,E) at 22°C are similar to wild-type embryos (A,D). At 28.5°C, *cyc* transcript levels are reduced in *cyc*^{sg1} mutant embryos at 70% epiboly (C), and not detected by 90% epiboly (F). Compared with wild-type embryos (G) or *cyc*^{sg1} mutant embryos at 28.5°C (H), *gsc* expression is variably reduced in *cyc*^{sg1} mutant embryos at 22°C (I,J).

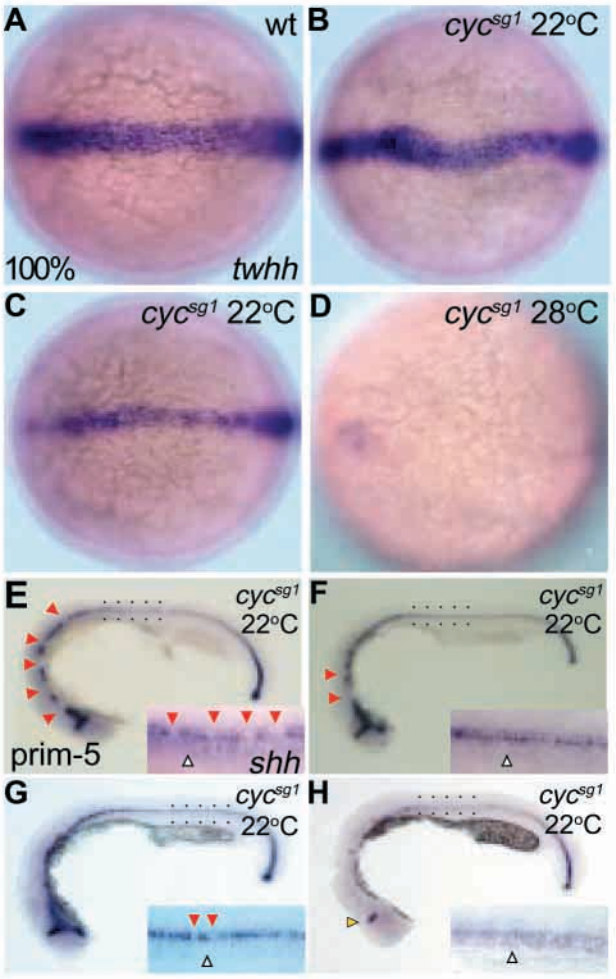

Fig. 4. Floor-plate cells are present in *cyc*^{sg1} mutant embryos at 22°C but not at 28°C. (A-D) Dorsal views; (E-H) lateral views with anterior towards the left. Expression of the early marker of floor-plate cells, *twhh*, in 100% epiboly wild type (A) and *cyc*^{sg1} mutants at 22°C (B,C) compared with lack of expression in *cyc*^{sg1} mutants at 28.5°C (D). At prim-5 stage, *cyc*^{sg1} mutants have patchy to complete *shh* expression in the floor plate at 22°C (E-G) and lack of floor plate *shh* expression at 28.5°C (H). (E) Gaps in the expression of *shh* in the ventral brain and spinal cord (red arrowheads). In E-H, dotted boxes mark the area displayed in the inset with patchy *shh* expression in the floor plate of the trunk and a normal notochord underneath (white arrowheads). (F) Gaps in *shh* expression in the hindbrain and rostral spinal cord (red arrowheads) but fairly complete floor plate in the trunk (inset). (G) Nearly complete ventral brain and rostral spinal cord floor plate, and patchy *shh* expression in the trunk (inset, red arrowheads). (H) At 28°C, similar to *cyc* null alleles, *shh* expression is seen in the notochord (white arrowhead), but not in the overlying neural tube except for a few cells in the dorsal midbrain (yellow arrowhead).

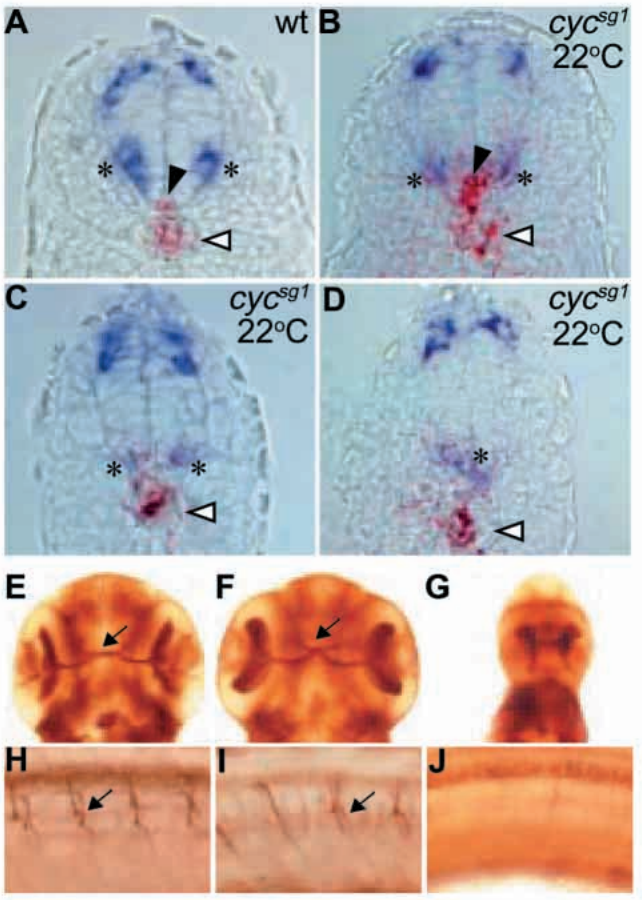

Fig. 5. Primary and secondary motoneurones in *cyc*^{sg1} mutant embryos. (A-D) Cross-sections at the level of the trunk. (E-G) Anterior views. (H-J) Lateral views of trunk. (A-D) Expression of *isl2* (purple, asterisks) marks the position of primary motoneurones in the ventral neural tube. Compare *shh* (red) expression in the floor plate (black arrowheads) and notochord (white arrowheads) in wild-type embryos (A) with that in *cyc*^{sg1} mutants at 22°C (B-D). Retinal ganglion cell axons (E-G) and secondary motoneurones (H-J) in wild type (E,H) or *cyc*^{sg1} mutants at 22°C (F,I) or 28°C (G,J). Black arrows indicate axonal projections from retinal ganglion cells and secondary motoneurones.

Continual Cyclops signalling is required during gastrulation for formation of a complete floor plate

To confirm the above observations that the floor-plate inducing activity of Cyclops is essential during mid-gastrulation, we addressed whether a transient pulse at the permissive temperature during mid-gastrulation was sufficient for induction of the floor plate, or, conversely, if a brief incubation at the restrictive temperature could abrogate floor-plate fates in *cyc*^{sg1} mutant embryos. When embryos were incubated at 28.5°C throughout gastrulation and segmentation, with a brief shift-down period at 22°C during mid-gastrulation, 27/29 mutant embryos (93%) that were incubated at 22°C between 70 and 80% epiboly showed rescue of medial floor plate cells, determined by the presence of *shh*- or *twhh*-expressing cells (Fig. 7A,D,H). However, the extent of rescue as determined by *shh* expression at prim-5 stage was usually patches of three or four cells, throughout the length of the neuraxis (Fig. 7H). If the pulse of 22°C was given between 60 and 90% epiboly, all

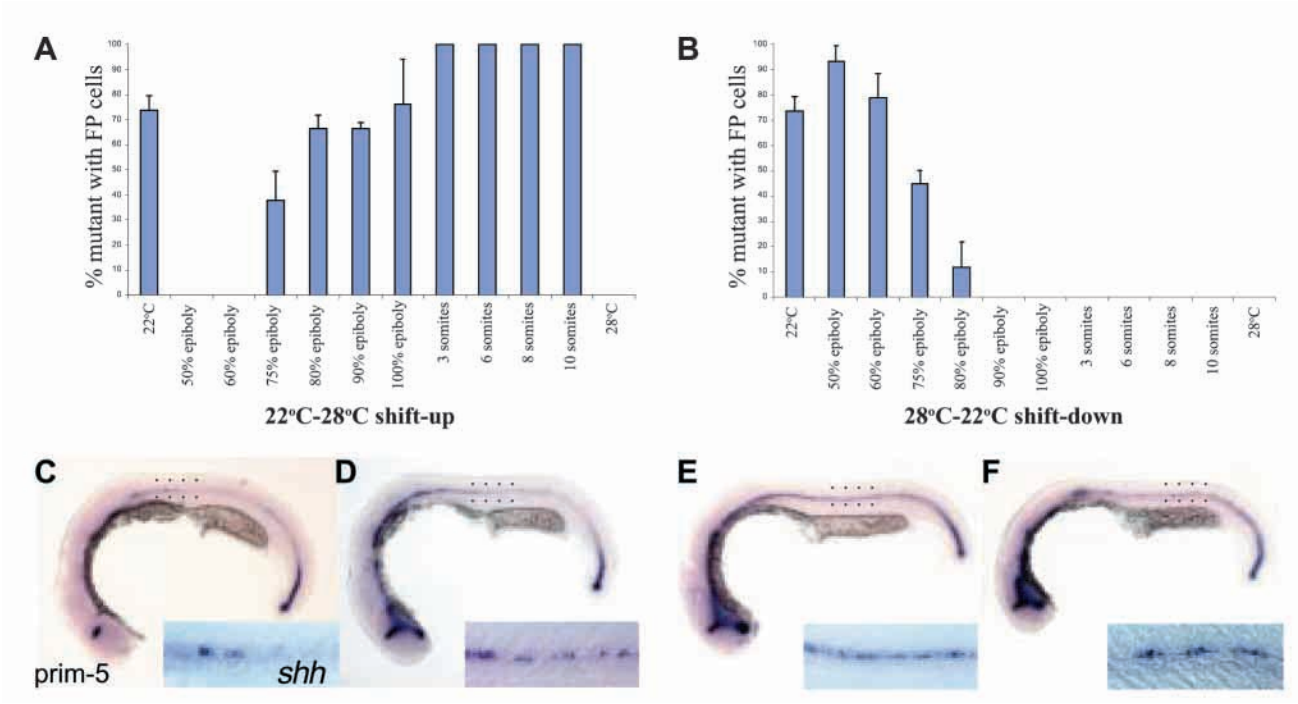

Fig. 6. Temperature shift experiments indicate that the floor plate is induced during gastrulation. (A) In shift-up experiments, embryos transferred from 22°C to 28.5°C after 75% epiboly show patches of *shh*-expressing (*shh*+) floor-plate cells (C), which increased significantly if the shift was performed at 80% epiboly and later stages (D). (B) In experiments where embryos were shifted down from 28°C to 22°C, embryos shifted at 50% epiboly and 60% epiboly had complete expression of *shh* in the floor plate (E). The number of embryos with *shh*+ floor-plate cells, and the extent of rescue, decreases if the shift-down is performed later during gastrulation (F). Lateral views are shown.

mutant embryos (*n*=17) showed floor-plate cells (Fig. 7A,B,F). Furthermore, the embryos showed longer stretches of cells expressing *shh* (Fig. 7F), with a complete floor plate in the trunk in all mutant embryos. Conversely, only 9/30 (30%) of the mutant embryos that were raised at 22°C and incubated at 28.5°C between 70 and 80% epiboly showed floor plate cells (Fig. 7A,E,I). The number of cells expressing *twhh* or *shh* were also fewer, with large gaps between patches of floor plate cells (Fig. 7E,I). If the pulse of 28.5°C was given between 60 and 90% epiboly, all mutant embryos (*n*=24) lacked floor plate cells (Fig. 7C,G). Thus, although a transient pulse of Cyclops signalling between 70-80% epiboly is sufficient to initiate floor plate fates, continuous Cyclops signalling is required between 60 and 90% epiboly for a complete floor plate.

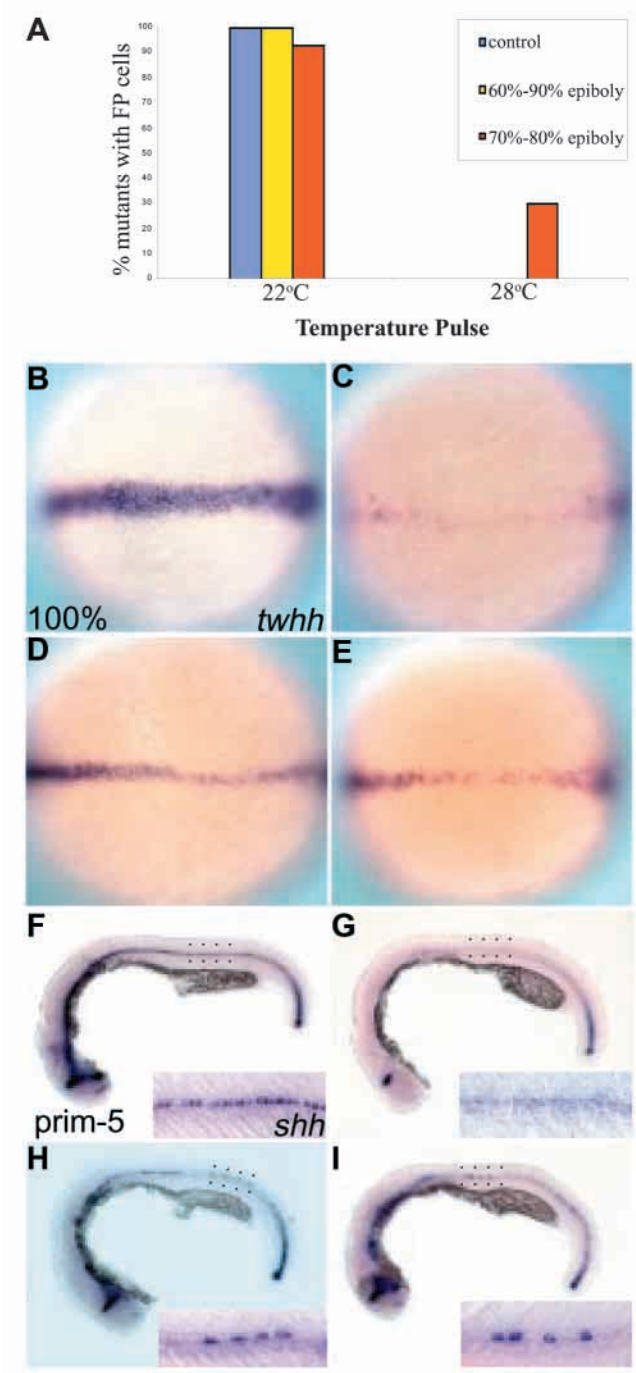

Fig. 7. Temperature pulse experiments reveal the precise time window of floor-plate induction. (B-E) *twhh* expression, dorsal view, (F-I) *shh*, lateral view. (A) While incubation at 22°C between 70 and 80% epiboly was sufficient to induce floor plate fates in >90% mutant embryos, the extent of rescue was groups of cells distributed throughout the neuraxis (D,H). Maximal rescue was observed in the embryos kept at 22°C between 60 and 90% epiboly (B,F). In the converse experiment, embryos pulsed at the restrictive temperature (28.5°C) showed very few (E,I) floor plate cells in the 70-80% interval or no floor plate cells (C,G) if pulsed at 28.5°C between 60 and 90% epiboly.

Induction and formation of a complete floor plate requires high levels of Cyclops signalling

Ectopic overexpression experiments in zebrafish and *Xenopus* have suggested that different levels of Nodal signalling pattern the organizer, with high levels required for specification of the anterior organizer fates (prechordal plate mesendoderm), and lower levels for posterior (notochord) fates (Jones et al., 1995; Gritsman et al., 2000). To determine if the level of Nodal signalling is important for induction of the floor plate, we overexpressed wild-type *cyc* mRNA in wild-type embryos. Although low doses of *cyc* RNA [or *squint* (*sqt*) RNA, data not shown] are sufficient to induce ectopic domains of the marker of the notochord, *flh* (Fig. 8B) (Gritsman et al., 2000) (*n*=147), expansion or duplication of the *gsc* and *twhh* expression domains (*n*=124 and 273, respectively) requires higher doses of *cyc* mRNA (Fig. 8G,H,K,L). Thus, high levels of Cyclops signalling are required for specification of floor plate fates.

This was supported by analysis of compound mutants of cyc^{sg1} generated with a null allele of *cyc*, cyc^{m294} and with the other zebrafish *nodal*-related mutant, sqt^{cz35}. Although all mutant embryos (38/186 *cyc*; genotype confirmed by PCR) from cyc^{sg1}/+ matings at 22°C had a complete floor plate (Fig. 9A), 4/79 embryos from matings of cyc^{m294}/+ with cyc^{sg1}/+ at 22°C had no *shh* expression in the floor plate (Fig. 9B), and

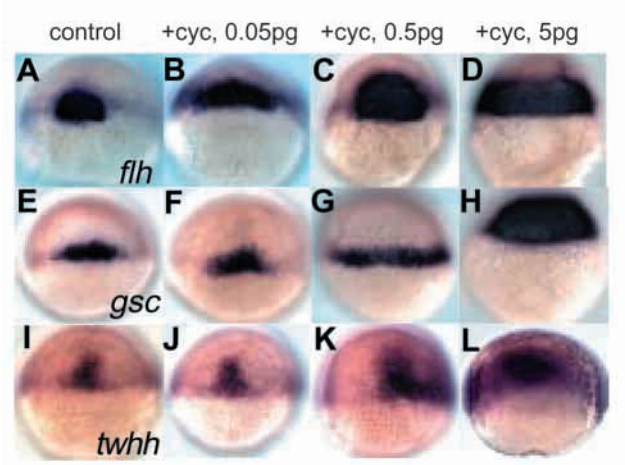

Fig. 8. Induction of the early floor-plate gene, *twhh*, requires high levels of Cyc/Nodal signalling. (A-L) Dorsal views at 50-60% epiboly. (A-D) *flh*; (E-H) *gsc*; (I-L) *twhh*. (A,E,I) Control embryos showing expression of all marker genes in the shield. Overexpression of 0.05 pg of cyc^{+} RNA results in expansion of the *flh* domain (B), but not of *gsc* (F) or *twhh* (J). Injections of higher doses of cyc^{+} RNA result in expansion of the *gsc* (G,H) and *twhh* (K,L) domains as well.

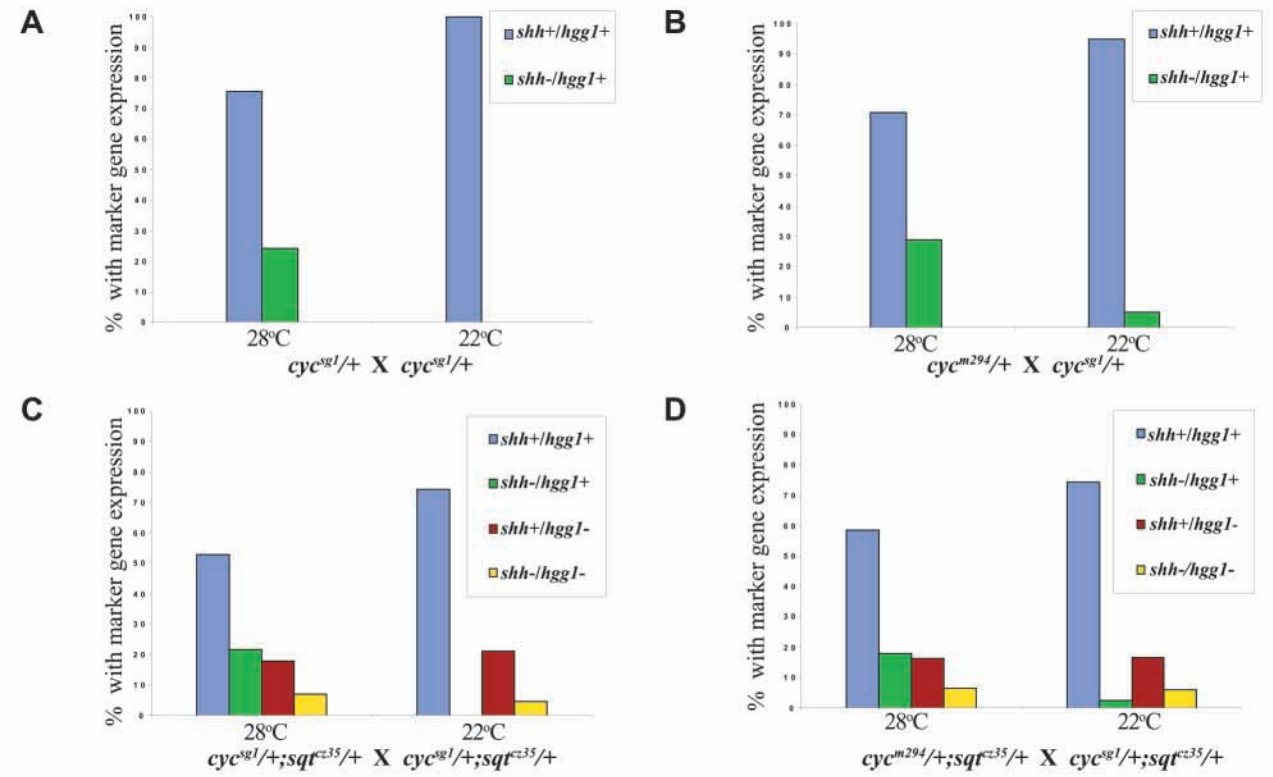

Fig. 9. Specification of floor-plate cells is dependent on high levels of Cyclops signalling. Embryos from matings of *cyc*^{sg1} (A), *cyc*^{sg1} with *cyc*^{m294} (B) and *cyc*^{sg1};*sqt*^{cz35} (C), and of *cyc*^{sg1};*sqt*^{cz35} with *cyc*^{m294};*sqt*^{cz35} (D) heterozygous fish were maintained at 22°C or 28°C, and analysed for *shh* expression to determine the extent of floor plate, and *hgg1* expression for the prechordal plate mesendoderm at prim-5 stage. (A) Mutants homozygous for *cyc*^{sg1} showed the expected proportion of embryos with *shh*- floor plate at 28°C, whereas at 22°C, all mutant embryos were *shh*+ for the floor plate through the entire length of the ventral neuraxis. (B) Embryos harbouring one null copy of *cyc* in combination with one copy of *cyc*^{sg1} showed a small proportion of mutant embryos with no *shh* expression in the floor plate domain, even at 22°C (4/79). However, the proportion of *shh*– embryos at 22°C was always less than that seen in the siblings from the same mating maintained at 28°C. In addition, 18 mutant embryos at 22°C showed patches of *shh*+ floor-plate cells. (C) In embryos from matings of *cyc*^{sg1};*sqt*^{cz35} transheterozygotes, at 22°C no *shh-hgg*+ embryos (genotype *cyc*) were detected, compared with 17% in the siblings kept at 28°C. In addition, the proportion of embryos that were lacking both *shh* and *hgg1* expression is significantly less at 22°C than that seen in embryos from the same clutches kept at 28°C. (D) In comparison with C, *shh-hgg1*+ embryos were seen in matings of *cyc*^{sg1};*sqt*^{cz35} with *cyc*^{m294};*sqt*^{cz35} heterozygotes, even at 22°C. In addition, the proportion of *shh–hgg*1– embryos was comparable with that seen in siblings from the same mating at 28°C.

18/79 embryos were *shh*+, but showed several gaps in *shh* expression in the floor-plate domain (genotype *cyc*; confirmed by PCR).

Similarly, Although 9/190 embryos (4.7%) at 22°C from *cyc*^{sg1}/+;*sqt*^{cz35}/+ matings did not express both *shh* and *hgg1*, a marker of the prechordal plate mesendoderm (genotype *cyc/cyc;sqt/sqt* double mutants, confirmed by PCR), clutches obtained from matings of *cyc*^{sg1}/+;*sqt*^{cz35}/+ and *cyc*^{m294}/+;*sqt*^{cz35}/+ fish showed a higher proportion (21/341; 6.2%) of *shh-;hgg1*- embryos (Fig. 9C,D) at 22°C. In addition, 68/254 embryos at 22°C from matings of *cyc*^{sg1}/+;*sqt*^{cz35}/+ and *cyc*^{m294}/+;*sqt*^{cz35}/+ were *shh*+*hgg*1+, but showed several gaps in the expression of *shh* in the floor-plate domain (genotype *cyc*; confirmed by PCR) (Fig. 9B,D). This is in comparison with no gaps in the floor-plate domain of *hgg1+;shh*+ embryos kept at 22°C from *cyc*^{sg1}/+;*sqt*^{cz35}/+ matings (36/141 *cyc*; genotype confirmed by PCR) (Fig. 9A,C). The floor plate in the trunk was also complete in all *hgg1-;shh*+ homozygous *sqt* mutant embryos (*n*=188). Similar results were obtained using the early markers, *twhh* and *gsc*, at 100% epiboly (data not shown). Therefore, although one copy of *cyc*^{sg1} is sufficient for the initial development of the floor plate from its precursors, it is not sufficient for a complete ventral neural tube along the length of the neural axis. Furthermore, deficiencies in the prechordal plate mesendoderm did not affect induction of the floor plate by Cyc^{sg1}.

DISCUSSION

cyc^{sg1} as a tool to understand the functions of Cyclops/Nodal signalling

Nodal signalling has been shown to be required for several patterning processes in early vertebrate embryos, ranging from the specification of mesoderm, endoderm and the ventral neural tube, to establishment of left-right asymmetry (Schier and Shen, 2000; Whitman, 2001). Many of these events occur early in development, and may be temporally or spatially overlapping, making it difficult to assess the precise requirements for Nodal signalling in each of these germ layers and processes. Evidence for the functions of Nodal signalling in specific tissues/germ layers has been obtained primarily

from the generation of chimeras in the mouse (Varlet et al., 1997; Brennan et al., 2001; Brennan et al., 2002). A hypomorphic allele has also been described in the mouse *nodal* gene (Lowe et al., 2001). However, the *nodal*$^{fl/\partial}$ mutant embryos die before gestation and manifest more severe phenotypes than our *cyc*^{sg1} homozygous mutant embryos at 22°C. Thus, the zebrafish temperature-sensitive *cyc*^{sg1}mutant provides a powerful tool with which to assess the precise requirements for Cyclops/Nodal signalling in all tissues and stages in which it functions.

Interestingly, the molecular lesion in *cyc*^{sg1} is a transversion which results in a premature stop codon in the pro domain. The receptor-binding functional moiety of TGFβ family proteins is thought to lie within the C terminus mature domain (Kingsley, 1994), and this region should be lacking in the Cyc^{sg1} mutant protein. However, *cyc*^{sg1} is functional at 22°C. Accordingly, we find that Flag-epitope tagged Cyc^{sg1} mutant protein is detected in zebrafish animal cap explants at 22°C, but not at 28.5°C. In addition, in Cos-7 cells, wild-type Cyc^{+} protein shows subcellular localization in a pattern reminiscent of the Golgi complex, whereas the mutant protein is not detected. Because an alternate start site (met 336) is present within the pro region after the stop codon, it is possible that the N terminus-truncated Cyc protein generated from this site is stable and functional in zebrafish embryos at 22°C but not at 28.5°C. Alternatively, a translational read-though mechanism similar to that described in mammalian cells (Laski et al., 1982; Hryniewicz and Vonder Haar, 1983; Phillips-Jones et al., 1995) may function at 22°C, the permissive temperature for *cyc*^{sg1}, allowing Cyc function at this temperature. It is also possible that the pro domain of Cyc has some activities that have not been previously identified. Analysis of Cyc^{sg1} can therefore provide valuable insights into the functions of various domains of Cyc/Nodal proteins.

Cyclops is required at multiple steps of floor-plate specification

By abrogation of Cyclops signalling in *cyc*^{sg1} temperature-sensitive mutant embryos at various stages of early development, we have determined that the crucial window for Cyc function in inducing the zebrafish floor plate is during gastrulation. Disruption of Cyc function during gastrulation by temperature shift experiments and modulation of the level of Cyclops signalling results in patchy or no medial floor plate marker gene expression. Interestingly, the *cyc*^{sg1} mutant embryos with patchy floor plate exhibit groups of floor-plate cells that are distributed throughout the length of the neuraxis. The presence of patches of floor plate cells throughout the length of the neuraxis suggests that the entire ventral neural tube arises from a group of precursors that are distributed throughout the length of the embryo, and that their differentiation into floor plate cells requires continuous Cyclops signalling during gastrulation. Previous observations by Hatta et al. (Hatta et al., 1991) where transplanted wild-type cells adopted floor-plate fates in *cyc* mutant hosts, and were able to recruit adjacent mutant host cells into floor plate fates, indicated that once specified, mutant cells had the ability to differentiate into floor plate cells. Our data indicates that sustained and high levels of Cyclops signalling are essential for the complete specification of floor-plate cells. Thus, in addition to being required for induction of cells of the floor plate and ventral neural tube, Cyclops signalling is also required for the development of a complete ventral neural tube throughout the entire length of the embryo.

Prechordal plate mesendoderm and the floor plate inducing activity of Cyc

Signalling in the anterior organizer cells, which give rise to the prechordal plate mesendoderm, has been implicated in induction of the floor plate (Sampath et al., 1998; Amacher et al., 2002). We find that deficiencies of the prechordal plate did not affect induction of the floor plate by Cyclops in *sqt*^{cz35}/*sqt*^{cz35}, *cyc*^{sg1}/*cyc*^{sg1};*sqt*^{cz35}/*sqt*^{cz35}, or *cyc*^{m294}/*cyc*^{sg1}; *sqt*^{cz35}/*sqt*^{cz35} mutant embryos. It is possible that the precursors of the prechordal plate cells or the remaining prechordal plate cells in these mutants are able to mediate floor plate induction via Cyclops signalling. Similar to the requirements for specification of prechordal plate mesendoderm (Gritsman et al., 2000), we find that uninterrupted and high levels of Cyclops signalling during gastrulation are crucial for induction and complete development of the floor plate in zebrafish. Sustained and high levels of Cyc/Nodal signalling during gastrulation can specify both floor plate and prechordal plate mesoderm fates. Therefore, we cannot rule out the possibility that it is the high level of Cyclops signalling, rather than signalling in the prechordal plate mesendoderm, which is responsible for floor plate induction.

Using the temperature-sensitive *cyc*^{sg1} allele, we have conclusively provided evidence that the medial floor plate is induced during gastrulation in zebrafish. It will be important to identify the cells in which Cyc/Nodal signalling is required for inducing the floor plate, the molecules that function downstream of Cyclops signalling to mediate this process in fish, and its similarities and differences with floor plate induction in amniotes. Given that several aspects of Nodal signalling are highly conserved (Schier and Shen, 2000), and given that axial/FoxA2/HNF3β is a common downstream effector of the floor-plate induction pathways in zebrafish as well as mice (Rastegar et al., 2002), similar mechanisms and timing of floor-plate induction are also likely in other vertebrates.

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