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Ectopic Wnt signal determines the eyeless phenotype of zebrafish *masterblind* mutant

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SUMMARY

***masterblind* (*mbl*) is a zebrafish mutation characterised by the absence or reduction in size of the telencephalon, optic vesicles and olfactory placodes. We show that inhibition of Gsk3β in zebrafish embryos either by overexpression of dominant negative dn gsk3β mRNA or by lithium treatment after the midblastula transition phenocopies *mbl*. The loss of anterior neural tissue in mbl and lithium-treated embryos is preceded by posteriorization of presumptive anterior neuroectoderm during gastrulation, which is evident from the anterior shift of marker genes *Otx2* and *Wnt1*. Heterozygous *mbl* embryos showed increased sensitivity to inhibition of GSK3β by lithium or dn Xgsk3β that led to the loss of eyes. Overexpression of gsk3β mRNA rescued eyes and the wild-type *fgf8* expression of homozygous *mbl* embryos. *emx1* that delineates the telencephalon is expanded and shifted ventroanteriorly in *mbl* embryos. In contrast to *fgf8*, the *emx1* expression domain was not restored upon overexpression of gsk3β mRNA. These experiments place *mbl* as an antagonist of the Wnt pathway in parallel or upstream of the complex consisting of Axin, APC and Gsk3β that binds and phosphorylates β-catenin, thereby destabilising it. *mbl* maps on LG 3 close to a candidate gene *axin1*. In *mbl* we detected a point mutation in the conserved minimal Gsk3β-binding domain of *axin1* leading to a leucine to glutamine substitution at position 399. Overexpression of wild-type *axin1* mRNA rescued mbl completely, demonstrating that mutant *axin1* is responsible for the mutant phenotype. Overexpression of mutant L399Q *axin1* in wild-type embryos resulted in a dose-dependent dominant negative activity as demonstrated by the loss of telencephalon and eyes. We suggest that the function of Axin1/Mbl protein is to antagonise the Wnt signal and in doing so to establish and maintain the most anterior CNS. Our findings provide new insights into the mechanisms by which the Wnt pathway generates anteroposterior polarity of the neural plate.**

Key words: *masterblind*, zebrafish, Wnt, GSK3β, lithium

INTRODUCTION

The primordium of the vertebrate central nervous system (CNS) acquires its anteroposterior (AP) polarity early during gastrulation (Houart et al., 1998). Studies in amphibian embryos have led to a two-signal model of AP patterning of the neural plate (Nieuwkoop et al., 1952). This model proposes that the first, activating signal, induces the neural tissue of the anterior character, the primordium of the forebrain. The second, transforming signal, imposes progressively more posterior fates onto the anterior neural tissue to generate the hindbrain and spinal cord. According to a recently proposed two-inhibitor model the anterior neural tissue is generated when Wnts and BMPs are simultaneously inhibited by Wnt and BMP antagonists (Niehrs, 1999) that are released by tissues such as anterior endoderm in the mouse (Beddington and Robertson, 1998), the prechordal plate in *Xenopus* (Glinka et al., 1998; Kazanskaya et al., 2000) or the yolk syncitial layer (ysl) and the prechordal plate of the zebrafish (Hashimoto et al., 2000). The idea is that the expression of Wnt inhibitors by axial mesoderm gradually decreases to the posterior, whereas expression of Wnts increases resulting in posteriorization of the neural tissue. Ectopic activation of the Wnt pathway during gastrulation inhibits formation of the head (Hoppler et al., 1996; Kim et al., 2000). Misexpression of Wnts in amphibia and fish after the MBT, leads to posteriorization of the CNS and may result in loss of the forebrain (Christian and Moon, 1993; Kelly et al., 1995; Fekany-Lee et al., 2000). In contrast, secreted Wnt antagonists such as Frzb (Leyns et al., 1997), Dkk1 (Glinka et al., 1998) and Wif1 (Hsieh et al., 1999) are capable of inducing secondary heads albeit in co-operation with BMP antagonists (Piccolo et al., 1999; Sirotkin et al., 2000).

The AP patterning of the neural plate also appears to be

tightly regulated by basal activity of Wnt pathway components that act to destabilise β-catenin. In the absence of Wnt signal, glycogen synthase kinase-3β (Gsk3β), Axin, the adenomatous poliposis coli (APC) protein and phosphoprotein phosphatase 2A (PP2A) form a complex that binds and phosphorylates β-catenin thereby stimulating its degradation by the ubiquitin-proteosome system (Aberle et al., 1997; Ikeda et al., 1998; Bienz and Clevers, 2000). In the presence of Wnts that trigger the canonical pathway, inhibition of Gsk3β activity allows the cytoplasmic accumulation of stabilised β-catenin. This unphosphorylated β-catenin then translocates into the nucleus where it modulates target gene transcription by interacting with members of the TCF/LEF1 family of transcription factors (Behrens et al., 1996; Molenaar et al., 1996).

In model organisms as well as in mammalian cells in culture, Gsk3β plays a key role in cell fate decisions by negatively regulating β-catenin (Welsh et al., 1996; Larabell et al., 1997). Lithium has been shown to inhibit Gsk3β, thereby mimicking Wnt signalling (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). Indeed, treatment of frog and fish embryos with lithium after the mid blastula transition (MBT) leads to posteriorization of the CNS that is strikingly similar to that induced by ectopic expression of Wnts (Yamaguchi and Shinagawa, 1989; Fredieu et al., 1997; Kelly et al., 1995). Consistent with the role of Wnt antagonist in anteriorization of the neural plate is targeted overexpression of gsk3β mRNA in *Xenopus* embryos that induces ectopic neural tissue of anterior character (Itoh et al., 1995; Pierce and Kimelman, 1996). Another negative regulator of the Wnt pathway is Axin that promotes Gsk3β-dependent phosphorylation of β-catenin thereby stimulating its degradation. Mutations in *axin*, such as those encoded by the *fused* locus in mouse and loss of function of *Drosophila axin*, phenocopy overexpression of Wnt/wg (Zeng et al., 1997; Hamada et al., 1999). The phenotype of *fused* homozygous mutants is characterised by axial duplications that are reminiscent of induction of the secondary axes in frogs by ectopic overexpression of Wnts (McMahon and Moon, 1989). Indeed, in the *Xenopus* axis-induction assay, *axin* inhibits induction of a secondary axis by negatively regulating Wnts (Zeng et al., 1997; Fukui et al., 2000). *fused* mutants also have neuroectodermal abnormalities which supports a role of *axin* in AP patterning of the CNS (Perry et al., 1995). Recently, genetic evidence has been presented to show that components of the Wnt pathway downstream of *axin* play a role in head induction. Severe head defects in the zebrafish *headless (hdl)* mutant are due to a loss of function of *tcf3* (Pelegri et al., 1998) that represses Wnt target genes (Kim et al., 2000).

masterblind (*mbl*) is a zebrafish recessive zygotic mutation from the Tübingen screen (Haffter et al., 1996). *mbl* is characterised by the absence or reduction in size of the telencephalon complemented by the anteriorward expansion of the epiphysis (Heisenberg et al., 1996; Masai et al., 1997). The phenotype of *mbl* suggests that the locus is required for normal development of the eyes and the telencephalon, the same structures that are negatively affected by ectopic Wnt signalling.

We present evidence that the function of *mbl* in normal development is to antagonise Wnt signalling and in doing so to establish and maintain the most anterior CNS. At the molecular and cellular levels, the posteriorised phenotype of *mbl* phenocopies the inhibition of Gsk3β in zebrafish embryos. By manipulating in embryos the dose of Gsk3β we show that the *mbl* mutant phenotype arises as a consequence of ectopic Wnt signalling. The defects in *mbl* were rescued by overexpression of *gsk3β* or *axin1* mRNA implicating that an ectopic Wnt signal underlies its phenotype. We detected in *mbl* mutants a point mutation in the minimal *gsk3β* binding domain of *axin1* (Heisenberg et al., 2001) that when injected into wild-type embryos had a weak dominant negative activity. Our findings provide new insights into the mechanisms that generate AP polarity of the neural plate and extend evidence for the crucial role of Wnt-pathway components such as *mbl/axin* therein.

MATERIALS AND METHODS

Fish lines

Zebrafish were maintained as described by Westerfield (Westerfield, 1995). Embryos were obtained by natural matings. Hubrecht Laboratory (HLwt) and Tübingen (Tüwt) wild-type fish strains were used for lithium experiments. *mbl* is a recessive lethal zygotic mutant (Heisenberg et al., 1996).

mbl (tm13) heterozygous fish were generated from *mbl* outcrosses in Tüwt background (a kind gift from Pascal Haffter, Tübingen) and subsequently in HLwt background.

Lithium treatments

Lithium (0.3 M LiCl) treatments were carried out on a shaking platform at 27°C in egg water (Westerfield, 1995) on sphere stage embryos (4hpf) (post-MBT treatment or lithium treatment). Treated embryos were subsequently washed 3 times in egg water and used in different experiments.

"Head" measurements (μm) of lithium-treated and wild-type embryos were carried out using an ocular micrometer in a dissecting microscope. Rostrocaudal distance was determined from the most anterior part of the head to the otic vesicles while dorsoventral distance was determined from the dorsal aspect of the yolk to the top of the head at the mid-hindbrain boundary.

Microinjection of synthetic mRNAs

One or 0.5 nl with identical end concentration of mRNA in water was injected into one or two-cell stage embryos with needles of approx. 5 μm diameter using a pneumatic picopump microinjector (WPI). Capped synthetic mRNAs were prepared using the SP6 mMessage mMachine kit (Ambion). The following constructs were used to prepare RNA for injection: full length (fl Xgsk3β), dominant negative (dn Xgsk3β), and frame shift *Xenopus gsk3β* (fs Xgsk3β) in pCS2+ linearized with *Not*I (kind gift from Dr Kimelman) (Pierce and Kimelman, 1995); zebrafish *gsk3β* linearized with *Not*I and zebrafish *axin1* linearized with *Asc*I (kind gift from Dr Hirano). In controls, equivalent quantities of GFP mRNA were injected and its expression assessed in living embryos.

DNA microinjection

Supercoiled plasmid DNA (1.4 nl of 40 μg/ml) for full length (fl *Xgsk3β*) or dominant negative (dn *Xgsk3β*) driven by the CMV promoter (kind gift from Dr Kimelman) (Pierce and Kimelman, 1995) was injected as previously described (Joore et al., 1996).

Whole-mount in situ hybridisation

Whole-mount in situ hybridisations were carried out as previously described (Joore et al., 1994).

Antisense DIG (Boehringer) labelled riboprobes were synthesised as described elsewhere: *otx2* (Li et al., 1994), *shh* (Krauss et al., 1993),

Krox20 (also known as *egr2*; Oxtoby and Jowett, 1993), *fgf8* (Reifers et al., 1998), *emx1* (Morita et al., 1995), *flh* (Talbot et al., 1995), *pax6* (Krauss et al., 1991), *wnt1* (Molven et al., 1991).

Embryo genotyping

To verify whether microinjections of mRNAs for gsk3β or *axin1* rescued the *mbl* phenotype we genotyped injected and control embryos. In PCRs we tested primer pairs for markers that are closely linked to the *mbl* locus, z15747, z24851 and z25683 on LG 3 (G.-J. Rauch and R. Geisler, pers. comm.). DNA samples of individual embryos were used for genotyping. Each 20 μl reaction contained 5 μl template DNA. The marker that showed the highest linkage to *mbl* was z24851 on 60.3 cM (purchased from MWG AG Biotech) **(**http://zebrafish.mgh.harvard.edu/cgi-bin/ssr_map/view_lg.cgi and http://wwwmap.tuebingen.mpg.de**)** and Fig.6.

Sequencing and *axin1*/*mbl* constructs

To identify the mutation in *axin1* causing the *mbl* phenotype, mRNAs isolated from *mbl* and wild-type embryos originating from two wild-type fish strains were used as templates in RT-PCR reactions. The following primers were used:

bp 6-734: gacagagtgcagggacactatgagc and aagactttgccgttgcc-tgacaccg

bp 498-1248: cgtatcccggcagatcaaacccg and cgcctctcgttccc-tcaacaccc

bp 947-1816: acgtgaactctggctacgcgctggc and tggtgttgggaccgac-gctcattcc

bp 1511-2076: gccattctccgaagtcccgctcg and ccccagacgctccacc-gaactgg

bp 1842-2645: ggcagcacgctatccaagcgaccg and tggctctccagcacgtcc-acgttcg

PCR reactions were performed using Amplitaq Gold (Perkin Elmer). Conditions were: 20 seconds 94°C, 20 seconds 67°C and 30 seconds 72°C.

PCR fragments were subsequently subcloned into pGEMT and sequenced in the presence of 1.3 M Betaine and 1.3% DMSO. The mutation found in *mbl* was confirmed by direct sequencing of the PCR product.

To make an *mbl* expression construct from which we made L399Q mutant *axin1* mRNA, a *Nsi*I-*Nar*I fragment from wild-type *axin1* was replaced by a *Nsi*I-*Nar*I fragment from *mbl* containing the mutation.

RESULTS

Lithium-mediated activation of the Wnt pathway induces posteriorization and concomitant loss of anterior brain

Lithium ions activate the Wnt pathway through inhibition of Gsk3β activity (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). When applied after MBT to zebrafish or *Xenopus* embryos lithium induces deletion of archaencephalic brain with the eyes being the most sensitive to its action (Stachel et al., 1993; Yamaguchi and Shinagawa, 1989; Fredieu et al., 1997).

To determine the sensitivity period for eye loss, embryos were exposed to lithium at different times after MBT. The most sensitive interval was from late blastula to early gastrulation, 4 to 6 hpf, when lithium induced loss of eyes or small eyes in almost 100% of embryos. During 7 to 8 hpf, susceptibility to lithium declined and progressively more embryos with small and wild-type eyes were observed (data not shown) (Stachel et al., 1993; Yamaguchi and Shinagawa, 1989).

The loss of anterior brain could be predicted at the tailbud stage by aberrant expression of neural plate regional marker genes. *wnt1*, a marker for the presumptive mid/hindbrain boundary, was expanded anteriorly in the apparently narrowed neural plate (Fig. 1A,B), while the expression of the midline marker *shh* was weaker in the anterior neural plate (Fig. 1C). Interestingly, the modification of *wnt1* expression in treated embryos was suggestive of abnormal convergent extension (CE) movements in the anterior neural plate (Fig. 1B). At 24 hpf the mild lithium phenotype was characterised by the loss of brain rostral to the D2/3 diencephalic boundary including the eyes, as revealed by loss of eye-specific *pax6* expression (Fig. 1D,E) and by an anterior shift of *otx2* (Fig. 1F). The severe phenotype was characterised by the loss of brain anterior to, and often including, the mid/hindbrain boundary as shown by loss of *Pax6* (Fig. 1D) and *Wnt1* expression (Fig. 1G). The hindbrain was unaffected as shown by expression of *Krox20* (Fig. 1H).

The lithium-induced deletion of the rostral brain was confirmed by comparison of the size of the head in wild-type and treated embryos at 24 hpf (see Materials and Methods). We found a reduction of approximately 15% of the rostrocaudal axis of treated embryos (515±55 μm, n=16) versus controls (694±16 μm, n=16), while the dorsoventral head dimension was unchanged.

To investigate whether the loss of brain induced by lithium was due to apoptosis, we carried out the TUNEL assay on lithium-treated tailbud and 24 hpf embryos**.** Since there was no difference in labelling between lithium-treated and wild-type embryos we conclude that apoptosis does not underlie the lithium phenotype (data not shown).

The data show that the loss of anterior brain caused by lithium-mediated ectopic activation of the Wnt pathway is the consequence of posteriorization of the neuroectoderm during early- to midgastrula.

Overexpression of dn Xgsk3β mRNA in wild-type embryos phenocopies *mbl* and lithium treatment

Lithium treatment inhibits Gsk3β activity and phenocopies the *mbl* defects. To test whether inhibition of Gsk3β activity is sufficient to phenocopy *mbl*, we overexpressed dn Xgsk3β mRNA (Pierce and Kimelman, 1996) in wild-type embryos with the expectation of inducing loss of eyes. Indeed, overexpression of dn Xgsk3β mRNA caused loss of eyes or reduction in their size in approx. 40% of injected embryos (n=154) (Fig. 2A,E). Although we titrated the mRNA concentrations to be injected and chose the concentration that induced the highest number of embryos with the eyeless phenotype associated with the lowest number of gastrulation phenotypes, there was still high mortality of injected embryos (Fig. 2A). It is likely that this mortality was caused by the disturbance of the Wnt pathway during axis formation (Harland and Gerhart, 1997; Moon and Kimelman, 1998). Consistent with this is the observation that a number of dn Xgsk3β mRNA-injected embryos displayed axial abnormalities at the tailbud stage as indicated by truncated and bifurcated expression of *flh*, the marker for the notochord anlage (not shown). To circumvent interference of the “early” Wnt pathway by dn Xgsk3β mRNA, we overexpressed the plasmid DNA containing dn Xgsk3β driven by the CMV promoter (1.4 nl of 40 μg/ml). In three independent experiments 13.5% (14/102) of embryos had loss of eyes and

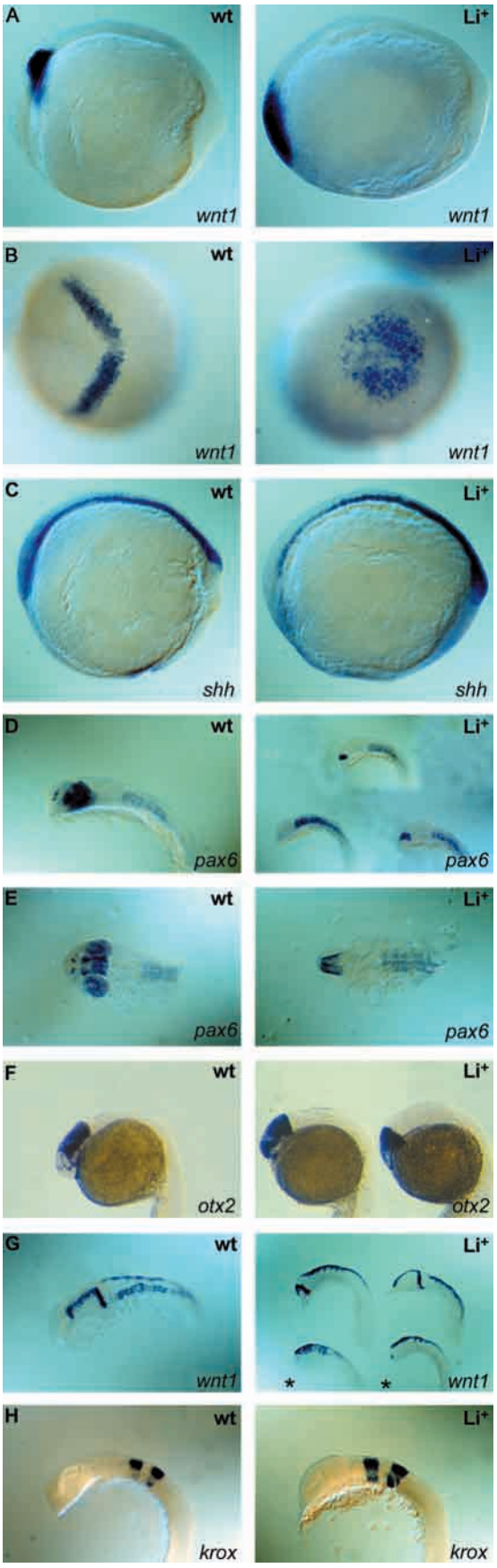

Fig. 1. Marker gene characterisation of "late" lithium phenotypes by in situ hybridisation of (A-C) tailbud stage and (D-H) 24 hpf (wild type, left) and lithium- treated (Li+, right) embryos (A,C,D,F,G,H, lateral view; B,E dorsal view; anterior to the left). (A,B) Anteriorly shifted *wnt1* covers the anterior neural plate (Li+). (C) *Shh* is diminished in the anterior midline (Li+). (D,F) The mild phenotype is characterised by the loss of brain anterior to D2/3 diencephalic boundary as revealed by (D) *pax6* and (F) *otx2*. (E,G) The severe phenotype is characterised by the loss of brain anterior to the MHB as revealed by (E) *pax6* and (G) *wnt1*. MHB was absent in the most severe cases (*). (H) The hindbrain was unaffected by the treatment as shown by *krox20* expression.

partial loss of forebrain. Since the efficiency with which dn Xgsk3β DNA overexpression induced the eyeless phenotype was very low, most likely due to mosaicism, we concluded that in our experimental design overexpression of dn Xgsk3β mRNA was to be preferred. The specificity of the observed effects of dn Xgsk3β mRNA on eye development was confirmed by injecting the inactive fs Xgsk3β mRNA (Pierce and Kimmelman, 1996), which caused a mutant eye phenotype in less than 5% of embryos (Fig. 2C). To study gain of function of gsk3β we overexpressed full length fl Xgsk3β mRNA and observed an eye/brain phenotype in approx. 10% of embryos (Fig. 2B) that was characterised by the deletion of the ventral forebrain and accompanying partial eye-fusion (Fig. 2F,G). Together these data suggest that the inhibition as well as ectopic activation of gsk3β signalling affects eye development.

To study whether inhibition of gsk3β in addition to eye development also interferes with morphogenesis of other brain compartments, we carried out in situ hybridisation with marker genes. Loss of function of *emx1* in the mouse influences telencephalic development (Yoshida et al., 1997). *emx1* in zebrafish is specifically confined to the dorsal telencephalon (Fig. 2H) (Morita et al., 1995). Overexpression of dn Xgsk3β mRNA did not affect *emx1* expression in 25/26 embryos with small eyes or in 8/39 eyeless embryos (Fig. 2J). In the one remaining embryo with small eyes an *emx1* expression domain was shifted anteroventrally as in *mbl* (Fig. 2I), whereas in the 31/39 eyeless embryos *emx1* was absent (not shown). Therefore, ectopic activation of the Wnt pathway through inhibition of Gsk3β may induce loss of eyes without affecting the telencephalon. However, when it induces deletion of the telencephalon the eyes are always lost as well.

In zebrafish, *fgf8* (*acerebellar*) is expressed at the MHB, in the dorsal diencephalon, retinae and optic stalks and in the facial ectoderm (FEC) (Fig. 2K; Reifers et al., 1998). Overexpression of dn Xgsk3β mRNA that induced small eyes in the majority of these embryos did not affect the *fgf8* pattern (16/24) (Fig. 2L, left panel). In the minority of embryos with small eyes the optic stalk labelling was absent (7/24) (Fig. 2L, right panel), while exceptionally (1/24), *fgf8* was expressed only at the MHB (not shown). In most of the embryos that lost their eyes upon dn Xgsk3β mRNA overexpression, *fgf8* was expressed at the MHB only (26/40) or not at all (14/40) (not shown). The data indicate that experimental activation of the Wnt pathway in zebrafish embryos through dn gsk3β overexpression can differentially affect telencephalon and eyes.

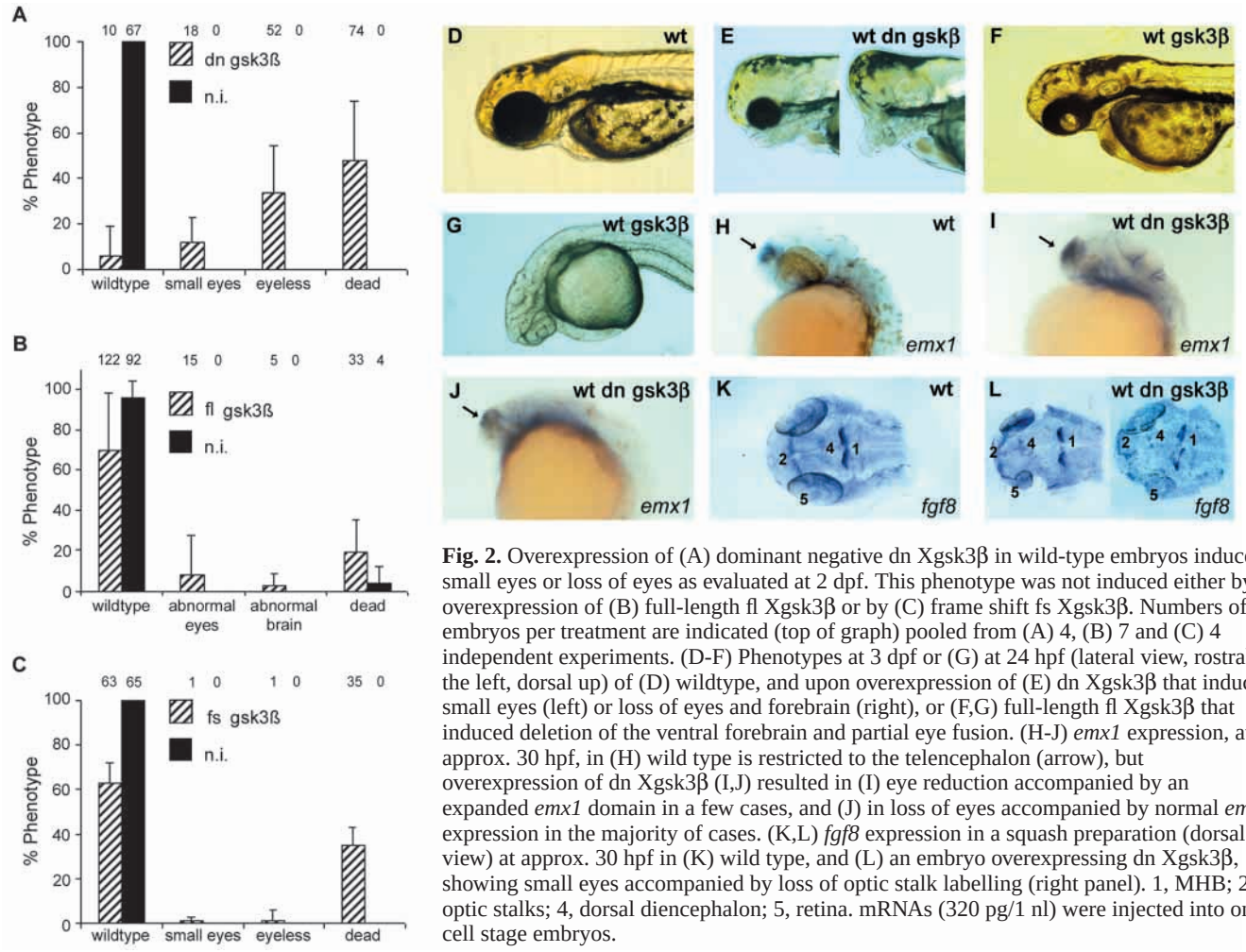

Fig. 2. Overexpression of (A) dominant negative dn Xgsk3β in wild-type embryos induced small eyes or loss of eyes as evaluated at 2 dpf. This phenotype was not induced either by overexpression of (B) full-length fl Xgsk3β or by (C) frame shift fs Xgsk3β. Numbers of embryos per treatment are indicated (top of graph) pooled from (A) 4, (B) 7 and (C) 4 independent experiments. (D-F) Phenotypes at 3 dpf or (G) at 24 hpf (lateral view, rostral to the left, dorsal up) of (D) wildtype, and upon overexpression of (E) dn Xgsk3β that induced small eyes (left) or loss of eyes and forebrain (right), or (F,G) full-length fl Xgsk3β that induced deletion of the ventral forebrain and partial eye fusion. (H-J) *emx1* expression, at approx. 30 hpf, in (H) wild type is restricted to the telencephalon (arrow), but overexpression of dn Xgsk3β (I,J) resulted in (I) eye reduction accompanied by an expanded *emx1* domain in a few cases, and (J) in loss of eyes accompanied by normal *emx1* expression in the majority of cases. (K,L) *fgf8* expression in a squash preparation (dorsal view) at approx. 30 hpf in (K) wild type, and (L) an embryo overexpressing dn Xgsk3β, showing small eyes accompanied by loss of optic stalk labelling (right panel). 1, MHB; 2, optic stalks; 4, dorsal diencephalon; 5, retina. mRNAs (320 pg/1 nl) were injected into one-cell stage embryos.

Common molecular mechanisms underlie *mbl* and lithium-generated phenotypes

To investigate whether a common mechanism underlies gsk3β inhibition and the *mbl* phenotype, we treated *mbl* mutants with a sub-effective dose of lithium. We reasoned that, owing to the dosage effect, *mbl* heterozygotes would be more sensitive to partial gsk3β inhibition than wild types. We used 5-10 minute exposure to lithium (Fig. 3). A 6 minute treatment applied to the offspring of $mbl^{+/-}$ outcrossed to HLwt, results in 50% eyeless embryos and 50% wild-type embryos (Fig. 3A). This same treatment induced less than 2% of embryos affected in wildtypes (Fig. 3B), demonstrating an enhanced sensitivity of *mbl* heterozygotes to ectopic activation of the Wnt pathway. Interestingly, the same treatment results in 25% small eyes, 25% eyeless and 50% wild types when the offspring of $mbl^{+/-}$ outcrossed to Tüwt is used (not shown). Therefore the HLwt background is more sensitive to gsk3β inhibition, possibly because of genetic differences in modifier genes that interact with the gsk3β signalling.

Our data show that lower levels of wild-type Mbl protein in *mbl* heterozygotes result in a higher sensitivity to gsk3β inhibition, indicating that *mbl* encodes a dosage-dependent inhibitor of Wnt signalling. These experiments support the hypothesis that gsk3β and Mbl function in the same pathway.

Overexpression of Xgsk3β rescues eyes of *mbl* embryos

To test the hypothesis that wild-type Mbl protein antagonizes the Wnt pathway we investigated the effects of injections of dn Xgsk3β or fl Xgsk3β mRNAs into progeny of in-crosses of *mbl* heterozygous fish. Overexpression of dn Xgsk3β mRNA induced loss or reduction of eyes in approx. 80% of embryos (Fig. 4A). When effects of dn Xgsk3β on wild-type embryos (Fig. 2A) as well as the increased susceptibility of heterozygous *mbl* embryos to lithium (Fig. 3A) are considered then 80% of embryos with a mutant phenotype would be predicted. To investigate whether the overexpression of fl Xgsk3β mRNA could rescue eyes in $mbl^{-/-}$ embryos, we injected this mRNA into the progeny of in-crosses of *mbl* heterozygotes. Because of the variable penetrance of the eye phenotype in *mbl* embryos that ranges from eyelessness to small eyes (Heisenberg et al., 1996), we present analysis of only eyeless progeny (Fig. 4B,D). Upon injection of fl Xgsk3β mRNA in embryos from eyeless clutches a reduction in eyeless embryos from 25% to 5% was accompanied by a corresponding increase in embryos with small eyes from 0% to 20% (Fig. 4B,E). These data strongly suggest that a simultaneous increase in the number of embryos with small eyes and a decrease of eyeless embryos

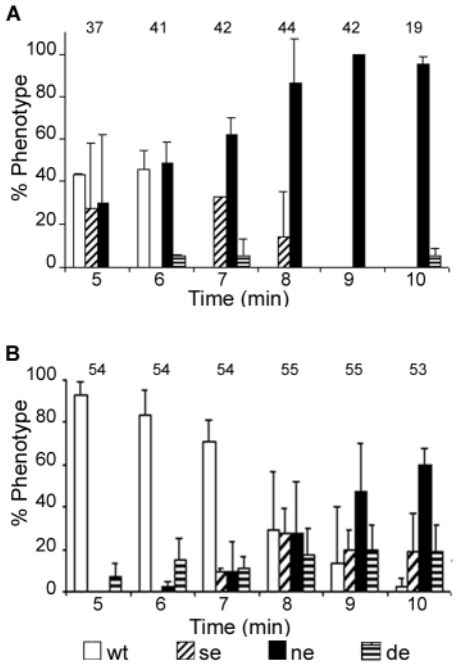

Fig. 3. Sensitivity to lithium of *mbl* heterozygotes as compared to wildtypes. Eye phenotype frequencies in 2 dpf zebrafish embryos treated at 4 hpf with lithium (0.3 M for 5-10 minutes). (A) Heterozygous *mbl*$^{+/-}$ × HLwt outcrosses. A 6-minute treatment resulted in 50% eyeless and 50% wild-type embryos. (B) Wild-type crosses where a 6-minute treatment resulted in a less than 2% phenotype. Numbers of embryos pooled from 2 independent experiments are indicated at the top of the graph. Wt, wild type; se, small eyes; ne, no eyes; de, dead.

reflects eye-rescue of homozygous eyeless *mbl*$^{-/-}$ embryos by fl Xgsk3β mRNA.

To confirm that phenotypically rescued embryos were indeed *mbl*$^{-/-}$, we genotyped uninjected and fl Xgsk3β mRNA-injected embryos. The marker closest to the *mbl* locus (G.-J. Rauch and R. Geisler pers. comm.) appeared to be z24851 on 60.3 cM (see Materials and Methods; Fig. 6) i.e. all phenotypically wild-type embryos (153/153) were genotyped as siblings (we could not discriminate between heterozygotes and wild types), 71/73 phenotypically mutant embryos were genotyped as *mbl*$^{-/-}$, whereas two eyeless embryos were genotyped as siblings (Table 1). Genotyping of fl Xgsk3β mRNA-injected embryos from eyeless clutches demonstrated that in 60% (18/29) of genotypically *mbl*$^{-/-}$ embryos eyes were rescued upon overexpression (Table 1). Surprisingly, 9/107 genotypic siblings had reduced eye size. Most of these embryos (7/107) originate from an experiment in which they were evaluated for marker genes prior to genotyping. Since the pigmentation was still weak and absolute eyesize difficult to determine in these embryos wild-type eyes may erroneously have been scored as small eyes. Moreover, some of the embryos with unexpected phenotypes may have recombinations between the marker and *mbl* locus. The genotyping confirmed our previous observation (Fig. 2B,F,G) that in the wild-type background gain-of-function Gsk3β leads to approx. 13% (Table 1) of embryos with the overexpression phenotype, i.e. the fused eyes and deleted ventral forebrain.

To confirm the validity of rescues mediated by fl Xgsk3β mRNA we injected zebrafish zf gsk3β mRNA into *mbl* embryos (kind gift from Drs T. Hirano/M. Hibi; Shimizu et al., 2000). As expected, since conservation at the protein level of gsk3β from the two species is high (>80%), fl zf gsk3β mRNA rescued eyes in *mbl*$^{-/-}$ equally well as fl Xgsk3β mRNA (data not shown).

To study whether the wild-type brain has been restored in fl Xgsk3β- rescued *mbl*$^{-/-}$ embryos with small eyes, we analysed expression of *flh*, *emx1* and *fgf*8. The zebrafish homeobox gene *floating head* (*flh*) that is essential in notochord development (Talbot et al., 1995) is required for progression of neurogenesis in the epiphysis. In *mbl*$^{-/-}$ embryos epiphysal neurons are generated throughout the dorsal forebrain within the ectopic *flh* expression domain that delineates the front of the neural plate as early as the tailbud stage (Fig. 4 G; Masai et al., 1997). Overexpression of fl Xgsk3β in *mbl* embryos resulted in a marked downregulation of this ectopic *flh*-domain at the anterior neural plate (Fig. 4H) that now resembled the wild-type expression (Fig. 4F) and most likely predicted eye rescue.

The telencephalic domain of eyeless and small eyes *mbl* embryos was shifted ventrally and anteriorly as revealed by an expanded *emx1* domain (Fig. 5B,C). These data suggest that telencephalic domain in *mbl* embryos is in molecular terms not lost, but transformed, with, as a consequence, the lack of differentiated telencephalon. The data show that overexpression of fl Xgsk3β may rescue eyes, without fully restoring the wild-type *emx1* domain (Fig. 5D). To further analyse the effect of rescue on brain regionalisation, *mbl* embryos were labelled with *fgf8*. In uninjected *mbl* embryos, the loss of eyes was accompanied by the disappearance of *fgf8* expression in the optic stalks and retinae and weakening or disappearance of the expression in the dorsal diencephalon, whereas expression at the mid/hindbrain boundary and in facial ectoderm was present (Fig. 5F). *fgf8* expression in rescued embryos with small eyes was restored to various degrees (Fig.

Table 1. Eye rescue upon overexpression of fl XGSK3β mRNA or *axin1/mbl* mRNA into progeny from *mbl*$^{+/-}$ incrosses

	flXGSK3β				*Axin1*			
	Not injected *n*=207		320 pg/nl or 650 pg/0.5 nl *n*=136		Not injected *n*=19		50 pg/nl *n*=34	
Phenotype	Sib%	Mut%	Sib%	Mut%	Sib%	Mut%	Sib%	Mut%
Wt	69	0	60.3	1.5	52.6		67.6	26.5
Se			5.9	6.6		42.1	0	5.9
Uni			0.7	3.6				
Ne	1	30	0	8.1		5.3		
Oe			11.8	1.5				

Wt, wild type; se, small eyes; uni, one small/one wild-type eye; ne, eyeless; oe, overexpression phenotype.

5G,H). In addition to the wild-type pattern (left eye in Fig. 5H), eye rescue could be established without restoration of *fgf8* in retinae (left eye in Fig. 5G), suggesting that Xgsk3β is sufficient to fully restore eyes in *mbl*$^{-/-}$ embryos.

mbl corresponds to *axin1*

The question of whether *mbl* corresponds to gsk3β is readily answered; the *mbl* locus does not encode gsk3β, since they map on different linkage groups, LG 3 and LG 9, respectively (Shimizu et al., 2000; http:/wwwmap.tuebingen.mpg.de). Our data strongly suggest that although the gsk3β-mediated rescue of *mbl* is incomplete it plays a pivotal role in the pathway involving wild-type *mbl* function. Since gsk3β antagonises the Wnt pathway through phosphorylation of β-catenin, it appears that a candidate gene for *mbl* should function similarly. *axin1* maps at 62.8 cM on LG 3 close to the marker z24851 at 60.3 cM that we found to be closely linked to *mbl*. *axin1* therefore represents an excellent candidate gene (Fig. 6A) (Geisler et al., 1999; Shimizu et al., 2000; Heisenberg et al., 2001). To test for the correspondence of *axin1* and *mbl* we sequenced *axin1* cDNA (accession no. AB032262; Shimizu et al., 2000) from 2 wild-type lines and a homozygous *mbl* mutant background. In *axin1* cDNA that was amplified from *mbl* mutants we found a 1 nt T → A transversion at position 1220 that results in a change of leucine into glutamine at position 399 in the protein (Fig. 6B). This leucine is conserved in a 25 aa sequence in *axin*, which represents the minimal gsk3β interaction domain (GID) that directly binds to gsk3β (Hedgepeth et al., 1999a). *axin1* mRNA is maternally present and its expression is ubiquitous in wild-type and *mbl* embryos until and including the tailbud stage (data not shown; Heisenberg et al., 2001). Importantly, at 24 hpf when the telencephalic domain is clearly delineated by *emx1* expression *axin1* mRNA appears to be concentrated in the midbrain and to be less abundant in the telencephalon, at the mid/hindbrain boundary (Fig. 6E) and throughout dorsal fore and midbrain. In *mbl* embryos there is an anterior brain domain that although misshapen, is similar to wild type in that it is characterised by low abundance of *axin1* message (Fig. 6F). To study the functional role of the leucine to glutamine mutation in GID of *axin*, we assessed the capacity of wild-type *axin1* mRNA to rescue *mbl*. Overexpression of 50 pg of full-length *axin1* mRNA resulted in more than 80% (9/11) phenotypic rescue of genotypically *mbl* embryos (Table 1). The *axin1*-mediated rescue frequently resulted in a wild-type phenotype, whereas gsk3β-mediated rescue most often resulted in smaller eyes. At higher concentrations overexpression of *axin1* induced a headless ventralised phenotype reminiscent of the phenotype of *sqt;boz* mutants (Sirotkin et al., 2000). Ectopic expression of an equivalent quantity of the mutant *mbl* RNA could not rescue the *masterblind* phenotype. Interestingly, in four independent experiments overexpression in wild-type embryos of 150 pg of mRNA transcribed from *axin1* containing the L399Q mutation resulted, at 24 hpf, in 25% (29/117) of embryos with a phenotype characterized by loss of telencephalon but with clearly present laterally positioned eyes (Fig. 6D), in 6% (7/117) eyeless embryos and 11% (13/117) dead embryos. These data suggest that upon overexpression, mutant *mbl* exerts a dose-dependent dominant negative activity, and provides in vivo evidence for the observation by Heisenberg et al. (Heisenberg et al., 2001) that TCF-dependent transcription in vitro is increased in the presence of L399Q mutant *mbl/axin*.

The data demonstrate that the *mbl* locus corresponds to *axin1* and that the recovered point mutation results in an altered *axin1* function that leads to activation of β-catenin /TCF.

In summary, mutation in minimal GID of *axin1/mbl* as well as a fine-tuned post-MBT treatment with lithium or injection of dn gsk3β mRNA all induce loss of eyes in zebrafish embryos, while overexpression of *gsk3β* or *axin1* rescues eyes of *mbl* embryos. The data are consistent with ectopic activation of β-catenin/TCF signalling in *mbl* embryos.

DISCUSSION

Lithium treatment that phenocopies *mbl* and *hdl* mutants blocks establishment of the anterior CNS through posteriorization of its progenitors

While lithium is known to exert effects on phosphoinositol metabolism (Berridge et al., 1989), recent evidence has demonstrated that its developmental effects are primarily due to activation of the Wnt pathway through inhibition of gsk3β and a consequent stabilisation of unphosphorylated β-catenin (Klein and Melton, 1996; Stambolic et al., 1996; Hedgepeth et al., 1997). When fish and frog embryos are treated with lithium post-MBT (present work; Yamaguchi and Shinagawa, 1989; Fredieu et al., 1997; Macdonald et al., 1994) loss of anterior CNS ensues that is reminiscent of phenotypes of the zebrafish mutant *masterblind* (*mbl*; Heisenberg et al., 1996). By treating embryos at different times during development with lithium we determined that the critical period for head induction is from late blastula (sphere) to early gastrula (shield) similar to what has been observed in *Xenopus* (Yamaguchi and Shinagawa, 1989). In order for normal brain development to proceed, Wnt signalling has to be excluded during this interval from the prospective forebrain region/the animal pole region (Woo and Fraser, 1995). The end of this interval coincides with the earliest patterning events within anterior neuroectoderm (Houart et al., 1997). It is likely that from this moment onwards ectopic Wnt does not interfere with head induction per se but instead disrupts genes that regionalise the neural plate. We show that deletion of anterior CNS in lithium-treated embryos is not due to apoptosis, but to posteriorisation of the anterior neuroectoderm as revealed by an anterior shift in expression of midbrain- and MHB-specific marker genes. An interesting proposition has been made that lithium posteriorises anterior neuroectoderm by negatively affecting cell proliferation which appears to be the highest in the anterior neural plate (Yamada, 1994; Gathpande et al., 1993; Hall, 1942). Based on our data that programmed cell death does not underly loss of anterior CNS in zebrafish embryos treated after the MBT with lithium and in conjunction with data from these classical experiments we speculate that the population of cells that would generate the forebrain in normal development never arises in lithium-treated embryos. Indeed, this may well be the reason why the anterior tissue of lithium-treated embryos is not only transformed to posterior fates, but is lacking all together. Therefore it may be that this ectopic Wnt signal, through

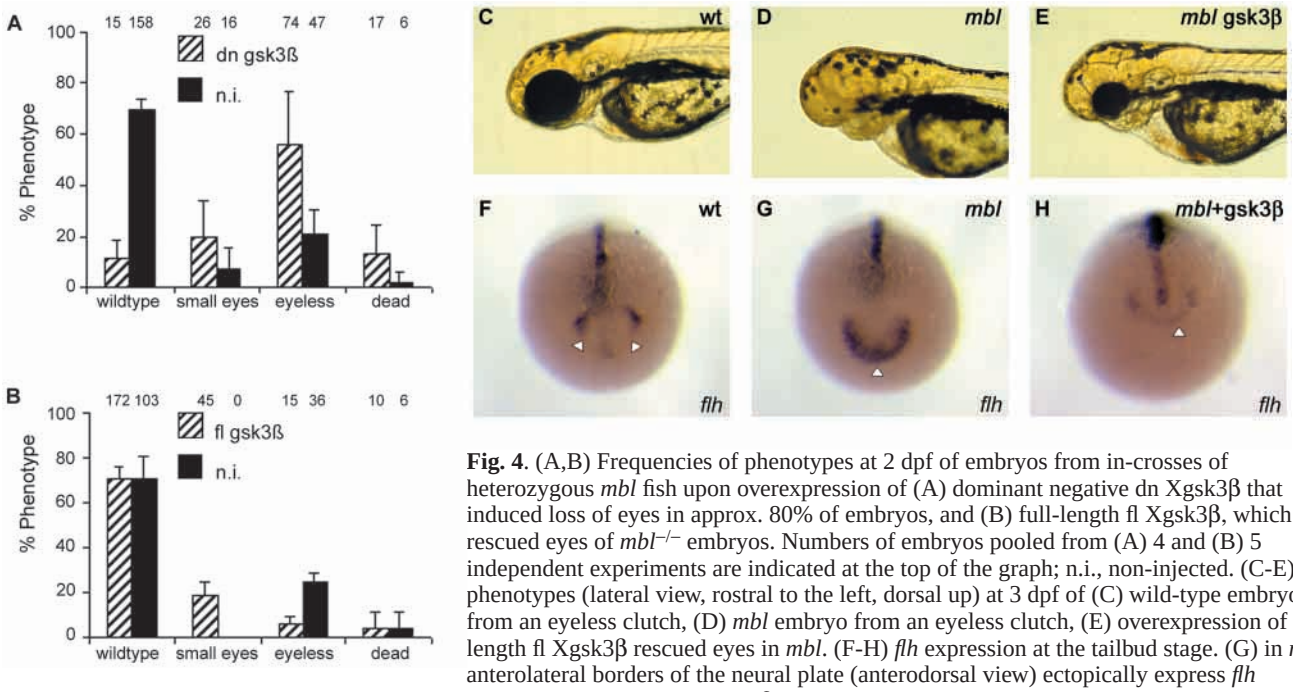

Fig. 4. (A,B) Frequencies of phenotypes at 2 dpf of embryos from in-crosses of heterozygous *mbl* fish upon overexpression of (A) dominant negative dn Xgsk3β that induced loss of eyes in approx. 80% of embryos, and (B) full-length fl Xgsk3β, which rescued eyes of *mbl*$^{-/-}$ embryos. Numbers of embryos pooled from (A) 4 and (B) 5 independent experiments are indicated at the top of the graph; n.i., non-injected. (C-E) Eye phenotypes (lateral view, rostral to the left, dorsal up) at 3 dpf of (C) wild-type embryo from an eyeless clutch, (D) *mbl* embryo from an eyeless clutch, (E) overexpression of full-length fl Xgsk3β rescued eyes in *mbl*. (F-H) *flh* expression at the tailbud stage. (G) in *mbl*, anterolateral borders of the neural plate (anterodorsal view) ectopically express *flh* compared to (F) wild type (arrowheads). (H) Overexpression of full-length fl Xgsk3β reduced ectopic expression of *flh* (arrowhead, ectopic *flh* in *mbl*). mRNA (320 pg/1nl) was injected into one-cell stage embryos.

activation of inappropriate target genes, downregulates proliferation. Although this proposition seems to contradict the well established role of Wnts as proto-oncogenes known to stimulate proliferation in cancer, the situation in the context of an embryo may be different. In the anterior neural plate, a balance of signalling inputs may result in a specific combination of activated target genes which accounts for inhibition rather than stimulation of cell cycling.

Interestingly, the block on gsk3β activity and a consequent activation of the Wnt pathway by the post-MBT lithium treatment, in the most severe cases, phenocopies zebrafish *hdl* (*headless*) mutants (Kim et al., 2000). The common molecular mechanisms underlying this phenotypic resemblance is evident since in *hdl* the loss of function of Tcf3 (Pelegri et al., 1998), a transcriptional repressor in the canonical Wnt pathway results in derepression of Wnt targets (Kim et al., 2000), while lithium treatment inhibits *gsk3β* and apparently activates an overlapping set of targets (Fredieu et al., 1997; this work). The phenotypes of *mbl* and *hdl* as well as of lithium-treated and dn gsk3β-injected embryos are presumably due to activation of Wnt target genes, which are repressed in the anterior CNS in normal development.

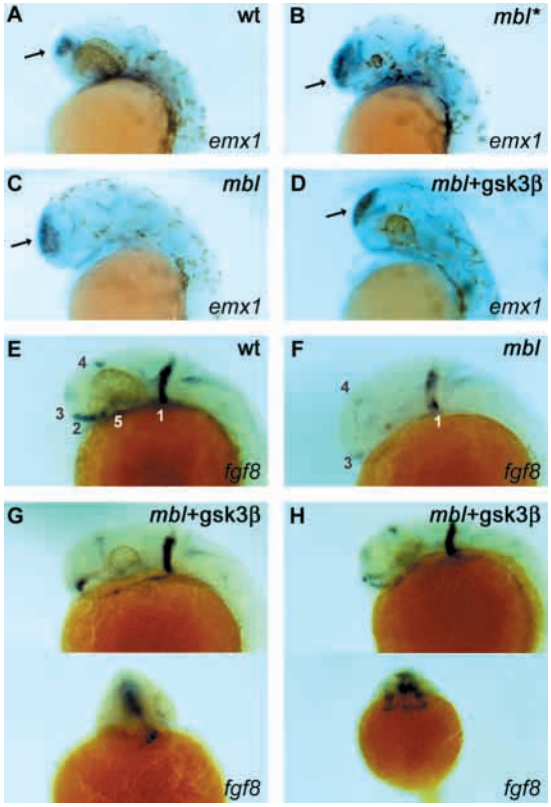

Fig. 5. *emx1* expression at approx. 30 hpf is restricted to the telencephalon in wild-type embryos (A). In *mbl* embryos with small eyes (B) and (C) eyeless, this expression is anteroventrally expanded. (D) Overexpression of full-length fl Xgsk3β in *mbl* embryos rescued eyes but did not normalize the mutant *emx1* domain. (E-H) *fgf8* expression at approx. 30 hpf in (E) wild-type embryos and (F) eyeless *mbl* embryos that lost the expression in retinae and optic stalks and still showed weak expression in facial ectoderm (FEC) and dorsal diencephalon and a normal expression at the MHB. (G,H) Overexpression of full-length fl Xgsk3β in *mbl* embryos rescued expression in one optic stalk and one eye (G) and (H) both eyes and fully rescued wild-type *fgf8* expression. (A-H, lateral view, rostral to the left; G,H lower panels in a frontal view.) 1, MHB; 2, optic stalks; 3, FEC; 4, dorsal diencephalon; 5, retina. mRNAs (320 pg/1nl) were injected in 1-cell embryos.

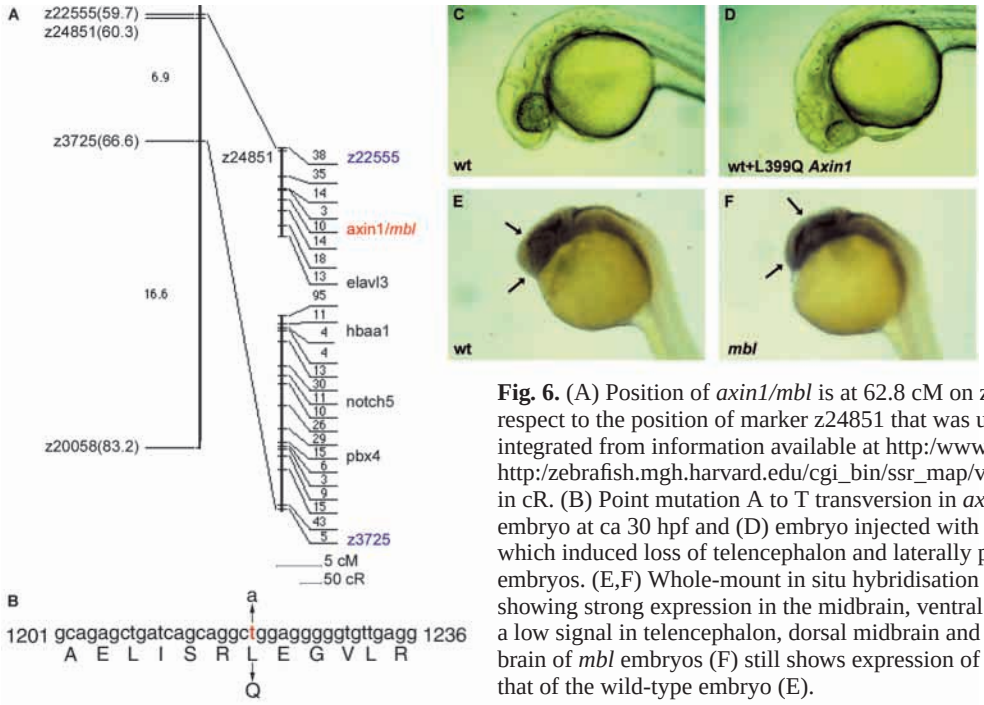

Fig. 6. (A) Position of *axin1/mbl* is at 62.8 cM on zebrafish LG 3 (59.7-83.2 cM) with respect to the position of marker z24851 that was used for genotyping. The LG 3 map was integrated from information available at http:/wwwmap.tuebingen.mpg.de and http:/zebrafish.mgh.harvard.edu/cgi_bin/ssr_map/view_lg.cgi. Left: scale in cM; right: scale in cR. (B) Point mutation A to T transversion in *axin1* of *mbl*. (C) Wild-type uninjected embryo at ca 30 hpf and (D) embryo injected with 150 pg of mutant L399Q *axin1* mRNA, which induced loss of telencephalon and laterally positioned eyes in 25% of 30 hpf embryos. (E,F) Whole-mount in situ hybridisation with *axin1* riboprobe in 24 hpf embryos showing strong expression in the midbrain, ventral midhindbrain and ventral hindbrain, and a low signal in telencephalon, dorsal midbrain and midhindbrain boundary. The abnormal brain of *mbl* embryos (F) still shows expression of *axin1* mRNA (arrows) comparable to that of the wild-type embryo (E).

Mutation in *mbl* and inhibition of gsk3β induce loss of eyes but have distinct effects on brain regionalisation

The posteriorising effect of an ectopic Wnt signal results in loss of eyes (Heisenberg et al., 1996; Masai et al., 1997; Kelly et al., 1995; Fredieu et al., 1997; Stachel et al., 1993; this work; Kim et al., 2000). Vertebrate eye formation is initiated by protrusion of optic vesicles from anterior neural tissue. The optic vesicle develops into the optic cup while inducing lens in the opposing ectoderm (Grainger, 1992). As a consequence of ectopic Wnt signal neither the optic vesicle nor the lens can form in *mbl* (Heisenberg et al., 1996). Our experiments show that this anterolateral region of the early neural plate that gives rise to optic vesicles is transformed in *mbl* embryos and now ectopically expresses *flh*. Accordingly, in *mbl* embryos rescued with *gsk3β* the wild-type *flh* pattern is restored. The optic vesicle presumably does not form because anterior neural tissue is transfated to posterior and has lost its morphogenetic properties. Interestingly, it has been suggested that overexpression of *gsk3β* in *Xenopus* interferes with signalling that controls morphogenesis of optic vesicles (Itoh et al., 1995). During early gastrulation this most anterior presumptive retina/forebrain field represents the source of the lens inducing planar signal (Grainger, 1992). Since in *mbl* embryos this region is posteriorised, the lens-inducing signals are likely not generated at all and as a consequence the lens fails to develop.

Although eye loss is common to *mbl*, *hdl* mutants, and experimental inhibition of *gsk3β*, the brainpattern is differentially affected. Although previous observations that in *mbl* embryos the telencephalon is reduced or missing are still valid (Heisenberg et al., 1996), we find that *emx1* expression, which in wild type delineates the most dorsal telencephalon, is in *mbl* not reduced or lost, but rather is expanded and shifted anteroventrally. It appears that the *emx1* domain is anteriorly displaced from its dorsal position as indicated by the diencephalic marker *flh* (compare Fig. 5B,C and Fig. 7B from Masai et al., 1997). The consequence appears to be the lack of differentiated telencephalon that in order to differentiate may require signals from the most anterior ventral CNS, which has been transformed in *mbl*. In contrast to *mbl*, overexpression of dn gsk3β either does not affect *emx1* expression at all, or in the most severe cases its domain is deleted together with all of the fore- and midbrain. However in 1 out of 26 embryos overexpressing dn gsk3β we observed the expanded *emx1* domain that is typical for *mbl* mutants. Moreover, our data show that overexpression of fl Xgsk3β in *mbl* mutants may rescue eyes, without restoring telencephalon, as evidenced by an aberrant *emx1* domain in these embryos that remains, as in mutant embryos, ventrally expanded**.** An exciting hypothesis is that eye and telencephalon would require different levels of Wnt inhibition for their development. To assess whether this hypothesis is valid we are currently investigating the localisation, abundance and phosphorylation status of β-catenin in *mbl* embryos as well as in embryos that received different experimental treatments.

The *mbl* locus corresponds to *axin1*

Our data suggest that interference with gsk3β signalling in the embryo results in different phenotypes as a consequence of quantitative differences in phosphorylation of β-catenin. Indeed, we show that the *mbl* locus signals in a dose-dependent fashion since a sub-threshold lithium treatment of *mbl* heterozygote embryos, which only marginally affected wild-type embryos, resulted in eyeless phenotype in heterozygotes. Conversely when *gsk3β* was overexpressed in homozygous *mbl* it was capable of rescuing eyes but not their wild-type size. We deduced that *mbl* contributes to Gsk3β-mediated phosphorylation of β-catenin. These experiments place the *mbl* gene in the canonical Wnt pathway in parallel or upstream of the complex consisting of APC, Gsk3β, PP2A

and β-catenin (Bienz and Clevers, 2000). Identification of *axin1* as the *mbl* locus (this work; Heisenberg et al., 2001) was therefore in agreement with the role proposed for *mbl*. *axin* was identified as the gene mutated in the *fused* mice that as homozygotes have axial duplications (Perry et al., 1995). Moreover, when tested in the *Xenopus* axis-induction assay, *axin* inhibits induction of a secondary axis by negatively regulating Wnt (Zeng et al., 1997; Fukui et al., 2000). In a multimeric complex, Axin simultaneously binds to APC, Gsk3β, PP2A and β-catenin to promote Gsk3β-mediated phosphorylation of β-catenin and its subsequent degradation (Bienz and Clevers, 2000; Ratcliffe et al., 2000). In this fashion *axin* prevents interaction of stabilised unphosphorylated β-catenin with TCF transcription factors and subsequent regulation of Wnt target genes (Roose et al., 1999; Bienz and Clevers, 2000). *mbl* is a point mutation in *axin1* resulting in a leucine to glutamine substitution at position 399 that leads to its loss of function. Leu 399 is conserved in a 25 aa sequence in Axin, which represents the minimal Gsk3β interaction domain (GID) that directly binds to Gsk3β (Hedgepeth et al., 1999b). The analogous mutation L525M in murine *Axin1* – present as L396M in human colorectal cancers – also interfered with gsk3β binding and resulted in low level activation of TCF-dependent transcription (Webster et al., 2000). Yet another L521P point mutation, also in GID of murine *axin1*, transformed it into a transcriptional activator (Smalley et al., 1999). Our data are in agreement with these findings, since we demonstrate that overexpression of *axin1* L399Q in wild-type embryos induces 30% phenotype most likely through its dose-dependent dominant negative activity. In contrast to the *mbl* phenotype, overexpression of *axin1* L399Q in wild-type embryos induces a deletion of the telencephalic region in a majority of embryos (80%), whereas only 20% becomes eyeless. The molecular basis for the dominant negative activity of *axin1* that is mutated in GID domain (Hinoi et al., 2000; Smalley et al., 1999; Webster et al., 2000) may be based on its interference with the function of wild-type *axin1*. In this scenario, mutant *axin1* would compete with wild-type *axin1* for components that form the multimeric complex with it, such as APC, PP2A and β-catenin. In this manner, mutant *axin1* would impair the function of wild-type *axin1*,which is to antagonise the Wnt signal. If true, those regions of the embryo containing lower levels of *axin1* mRNA would, upon overexpression of mutant *axin1*, be more sensitive to its dominant negative activity. Strikingly, zebrafish telencephalon contains less *axin1* mRNA than the midbrain (Fig. 6E,F.) It is therefore tempting to speculate that the telencephalon would be the most sensitive target to phenotypic alteration by dominant negative activity of overexpressed mutant *axin1*. Our experiments appear to support this possibility.

With regard to the *mbl* phenotype, it is interesting to note that *fused* homozygous mice display strong neuroectodermal abnormalities (Perry et al., 1995). Expression of *axin1* in the anterior midbrain of *Xenopus* embryos (Hedgepeth et al., 1999a) its anterior overlap at the mid/forebrain boundary with *tcf4* and its dorsal overlap with *wnt1* and *wnt3A* (Konig et al., 2000) a), as well as its strong expression in the midbrain of the zebrafish embryo (Fig. 6E,F) further support its role in regionalisation of the anterior brain.

Why does overexpression of gsk3β only rarely result in full phenotypic rescue of *mbl*?

Overexpression of *axin1* more efficiently and more completely rescues *mbl* than *gsk3β* overexpression (Table I), although in both cases rescue is expected to arise as a consequence of β-catenin phosphorylation and, as a result, counteraction of the ectopic Wnt signal. It has been shown that β-catenin is not a good substrate for Gsk3β in vitro, but is efficiently phosphorylated in the presence of Axin1 (Ikeda et al., 1998). Since the point mutation in GID of *mbl/axin1* abolishes binding of gsk3β to Axin1 (Heisenberg et al., 2001), this is likely to be the limiting factor for its enzymatic activity. We speculate that overexpression of gsk3β can partially overcome this limitation and to an extent rescue the *mbl* phenotype because it may very efficiently interact with maternal *axin1*. For instance, it has been shown in *Xenopus* egg extracts that the inhibitory effect of GBP and *dsh* on β-catenin degradation is reversed by high concentrations of gsk3β due to titration of GBP and *dsh* on *axin* (Salic et al., 2000). The same work suggests that the interaction between *axin*- gsk3β is highly dynamic since dn gsk3β blocks degradation of β-catenin in extracts. If so, then overexpression of *gsk3β* in *mbl* embryos would shift the binding equilibrium towards *gsk3β – axin*, and, as a consequence, degradation of β-catenin. The reason for the incomplete phenotypic rescue of *mbl* may be the limiting concentrations of maternal *axin1*. Alternatively, it is possible that the weak dominant negative activity of L399Q mutant *axin1* cannot be overruled by overexpression of gsk3β.

Does a gradient of Wnt signalling pattern the neural ectoderm?

Comparison of loss-of-function *hdl/tcf3*, lithium treatment, overexpression of dn gsk3β and loss of Gsk3β binding activity of *mbl/axin1* reveals that although they all activate an ectopic Wnt signal their resulting phenotypes differ. This may be due to the type of Wnt target genes and/or the extent to which they are ectopically activated. Expression patterns of Wnts and their regulators suggest that there is an AP gradient of activation/repression of Wnt targets in the anterior neural plate.

For instance, in the most severe phenotypic category of *hdl/tcf3*, dn gsk3β overexpression and severe lithium phenotype, Wnt target genes that during normal development are activated at the MHB and/or posterior to it, are in mutant and treated embryos derepressed and ectopically expressed anterior to the MHB (present work; Kim et al., 2000). During normal development repression of these genes in the most anterior domain secures head induction. The most anterior neuroectoderm is characterised not only by the highest expression of *tcf3*, the absence of Wnt ligands and high levels of *axin1/mbl* in the midbrain caudally to it, but is also exposed to secreted Wnt antagonists such as *dkk1* (Hashimoto et al., 2000) and perhaps others as well. These are only a few molecules among those that play a role in establishment of a gradient of Wnt signal. At the present moment the challenge is to identify expression and function of other potential players that generate neuroectodermal polarity, both known ones such as APC and PP2A and novel ones. Moreover, it should be possible, using specific antibodies, to carry out spatiotemporal analysis of stabilised unphosphorylated β-catenin and thus investigate whether there exists an AP gradient of Wnt signalling.

Since phosphorylation of β-catenin represents an integrating point of numerous input signals within the Wnt pathway we propose that there may be a gradient of constitutive inhibition (phosphorylation of β-catenin) with its apex at the anterior pole of the embryo. This gradient acts upon the posteriorising signal, which is the unphosphorylated β-catenin with the apex at the posterior pole.

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