Comparative synteny cloning of zebrafish *you-too*: mutations in the Hedgehog target *gli2* affect ventral forebrain patterning

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Zebrafish *you-too* (*yot*) mutations interfere with Hedgehog (Hh) signaling during embryogenesis. Using a comparative synteny approach, we isolated *yot* as a zinc finger transcription factor homologous to the Hh target *gli2*. Two alleles of *yot* contain nonsense mutations resulting in carboxy-terminally truncated proteins. In addition to causing defects in midline development, muscle differentiation, and retinal axon guidance, *yot* mutations disrupt anterior pituitary and ventral forebrain differentiation. *yot* mutations also cause ectopic lens formation in the ventral diencephalon. These findings reveal that truncated zebrafish Gli2 proteins interfere with Hh signaling necessary for differentiation and axon guidance in the ventral forebrain.

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Members of the Hedgehog (Hh) family of signaling molecules are secreted by midline cells that influence the differentiation of adjacent cells (Placzek 1995; Hammerschmidt et al. 1997; Dodd et al. 1998; Ingham 1998). Mutational analysis in mouse and zebrafish has established the essential role of *sonic hedgehog* (*shh*) in development. Mouse mutants lacking *shh* have ventral forebrain defects, lack a floor plate and motor neurons in the neural tube, and have reduced sclerotomal tissue in the somites (Chiang et al. 1996). Zebrafish *sonic-you* (*syu*) mutations disrupt the *shh* gene and lead to defects in the spinal cord, somites, and optic chiasm (Brand et al. 1996; van Eeden et al. 1996; Schauerte et al. 1998).

Genetic screens have identified several additional zebrafish mutations that have phenotypes similar to *syu*, and these may define additional components of the Hh signaling pathway (Brand et al. 1996; Karlstrom et al. 1996; van Eeden et al. 1996). In particular, *you-too* (*yot*) mutants lack an optic chiasm and a horizontal myoseptum in the somites. Overexpression of Shh fails to rescue the somite defects in *yot* (Schauerte et al. 1998). Furthermore, *yot* is required cell autonomously in the somites for proper muscle differentiation (van Eeden et al. 1996). These observations suggest that *yot* encodes a downstream component of the Shh signaling pathway.

We report here that *yot* mutations affect ventral forebrain development and disrupt zebrafish Gli2, a member of a family of zinc finger containing transcription factors implicated in mediating Hh signaling (Ding et al. 1998; Matise et al. 1998; Ruiz i Altaba 1998).

Results and Discussion

We mapped *yot* to linkage group 9 (LG9) of the zebrafish genetic map (Fig. 1A,B). Recent analysis revealed conserved synteny (gene linkage) between zebrafish LG9 and human chromosome 2 (Postlethwait et al. 1998). A survey of human chromosome 2 identified the zinc finger transcription factor *GLI2*, suggesting that a zebrafish *gli2* gene might reside on LG9 (Fig. 1A). *yot* mutations affect tissues known to require Hh signaling for proper differentiation, and *gli* genes [*Drosophila cubitus interruptus* (*ci*), vertebrate *gli1* and *gli2*] have been implicated in transducing Hh signals from the midline (Ruiz i Altaba 1997; Ding et al. 1998; Ingham 1998; Matise et al. 1998 and references therein). Thus, *gli2* was a compelling candidate for the *yot* locus. We therefore cloned zebrafish *gli2* and found that it resides on LG9 near *yot* (Fig. 1). The zebrafish *gli2* cDNA contains a full-length open reading frame (ORF) of 1440 amino acids that is closely related to mouse *Gli2* (Fig. 2). Sequencing revealed that both *yot* alleles contain nonsense mutations (Fig. 2B) predicted to result in carboxyl terminally truncated Gli2 proteins (Fig. 2C), confirming that the *yot* locus encodes Gli2.

The truncated Gli2 proteins retain the zinc finger DNA-binding domain but lack a region similar to a domain in *Drosophila* Ci implicated in binding dCBP, a transcriptional coactivator (Akimaru et al. 1997). They also lack a proposed VP16-like activation domain required for human Gli1 activity (Yoon et al. 1998). Because such carboxy-terminal deletions impair the ability of Ci and Gli1 to activate Hh targets (Alexandre et al. 1996; Yoon et al. 1998), the *yot* mutant proteins might not mediate full transcriptional activation in response to Hh signaling. The truncated Gli2 proteins share striking similarity to forms of Ci protein (Fig. 2C) that transcriptionally repress Hh target genes and form by post-translational processing in the absence of Hh signaling (Aza-Blanc et al. 1997). In addition, the *yot* truncations resemble potential repressor forms of Gli3 (Fig. 2C) that lead to dominant Pallister–Hall syndrome (PHS) (Kang et al. 1997) and postaxial polydactyly type A (PAP-A) (Radhakrishna et al. 1997). Taken together, these observations suggest that these proteins might act as repressors that form even in the presence of Hh and interfere

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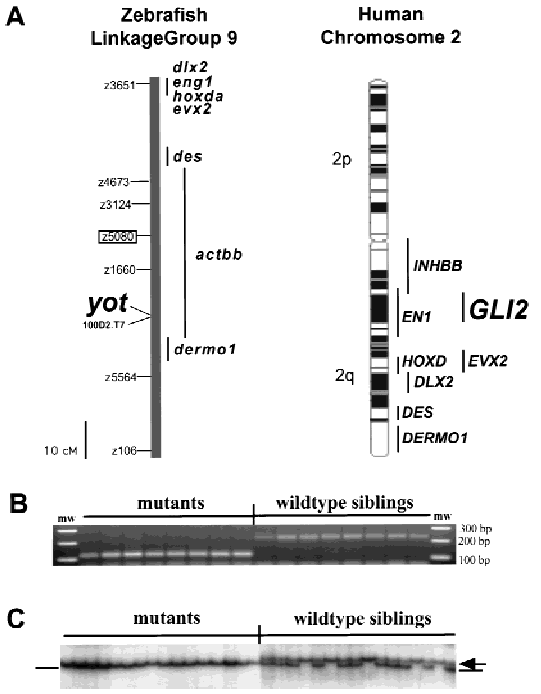

Figure 1. (*A*) Genetic map of linkage group 9 (LG9) showing position of *yot*/*gli2* in relation to previously mapped genes (Postlethwait et al. 1998) and SSLP markers (Knapik et al. 1998). yot^{ty17} was mapped to LG9 using centromere linkage analysis with centromeric marker z5080 (boxed) (see *B* and Materials and Methods), followed by mapping in a standard sexual intercross with marker z3124 (6 recombinants in 44 meioses). To determine the position of *yot* more precisely, we scored a marker that cosegregates with *yot* (BAC 100D2, see below) along with SSLP markers z3124, z5080, and z5564 on a haploid mapping panel (M.A. Gates, A.F. Schier, W.S. Talbot, in prep.). *yot* maps to the same linkage group as the zebrafish *hoxda* cluster (Amores et al. 1998), *dlx2*, *engrailed1* (*eng1*), *evx2*, *desmin* (*des*), *dermo1*, and *activinbb* (*actbb*). The orthologs of these genes are located on human chromosome 2, where *gli2* is also located. Human cytological map and locations were derived from Genome DataBase (http://gdbwww.gdb.org). Zebrafish *actbb* corresponds to human *inhibinbb* (*INHBB*). (*B*) Mapping to LG9 using SSLP marker z5080. z5080 primers amplified an ~120-bp fragment from yot^{ty17} DNA but an ~220-bp band from wild-type siblings, indicating linkage to the marker. No recombinants were seen in 16 individuals. (*C*) SSCP analysis links BAC 100D2 DNA to *yot*. PCR amplification of DNA from individual mutant embryos produced a faster migrating band (lines), whereas DNA from wild-type siblings produced a slower migrating band (homozygotes, arrow) or both bands (heterozygotes). No recombinants were seen in 230 mutant embryos, indicating tight linkage.

with Hh signaling. Supporting this possibility, previous studies have shown that *yot* mutations block transmission of Hh signaling in target tissues (van Eeden et al. 1996; Schauerte et al. 1998). The weak dominant effects on somite patterning found in *yot* heterozygous embryos (van Eeden et al. 1996) are also consistent with the idea that *yot* mutations encode repressor forms of Gli2.

To determine if *gli2* expression domains correspond to the regions affected by mutations in *yot*, we performed *gli2* RNA in situ hybridization (Fig. 3). *gli2* transcripts are first detected in the anterior neural plate and in somitic mesoderm precursors that are affected in *yot* mutants (Fig. 3A,B). As development proceeds, *gli2* is expressed throughout the dorsal forebrain, midbrain, and hindbrain and is generally expressed dorsal and adjacent to cells that express *shh*. In the postoptic area of the ventral diencephalon, however, *gli2* and *shh* expression overlap (Fig. 3E,G,H). This *gli2*-expression domain corresponds to the site of axon growth affected in *yot* mutants (see below) and is consistent with a direct role of *gli2* in the formation of this area of the ventral diencephalon.

Although *gli* genes have been implicated in forebrain development (Lee et al. 1997; Ruiz i Altaba 1998), the consequences of *gli* mutations on ventral forebrain patterning have not been analyzed. To define ventral forebrain defects in *yot* mutants more precisely, we first examined axon guidance and cell differentiation in this area. In the ventral diencephalon of *yot* mutant embryos, the formation of the optic chiasm (Brand et al. 1996; Karlstrom et al. 1996) and postoptic commissure are both affected (Fig. 4). Instead of crossing the midline in the postoptic area (Fig. 4C), retinal axons grow dorsally immediately after leaving the eye (Fig. 4D). The earlier forming postoptic commissure also fails to form in *yot* mutant embryos, and the anterior commissure in the telencephalon is reduced (Fig. 4B). Further, lens-like structures form in the anterior pituitary region of the ventral diencephalon (Fig. 4D) in nearly all yot^{ty119} mutants and in 10%–50% of yot^{ty17} mutants. These results establish that *yot* mutations lead to defects in axon guidance and cell differentiation in the ventral diencephalon.

The mis-specification of ventral forebrain tissue, along with forebrain expression of *gli2*, suggests that axon guidance defects in *yot* mutants might be caused by abnormal development of ventral forebrain cells that serve as substrates or guidance cues for navigating axons. To test this idea, we examined the expression of ventral forebrain markers in *yot* mutant embryos. The homeobox gene *nk2.2* is normally expressed by cells in the rostral part of the anterior pituitary anlage and in the postoptic area (Fig. 5A; Barth and Wilson 1995). In *yot* mutants, the rostral anterior pituitary expression of *nk2.2* is absent (Fig. 5B). The homeobox gene *lim3*, a marker of the anterior pituitary anlage (Glasgow et al. 1997), is also reduced in the region of the anterior pituitary in *yot* mutants (not shown). *six3*, a homolog of *Drosophila sine oculis*, is a homeobox gene expressed in the anterior neural plate, anterior pituitary, and the hypothalamus (Fig. 5C; Kobayashi et al. 1998; Seo et al. 1998). In *yot* mutant embryos, *six3* expression is absent from the rostral part of the anterior pituitary and the adjacent domain of the postoptic area but remains in the anterior postoptic area and caudal part of the anterior pituitary (Fig. 5D). The defects in the expression of *nk2.2*, *lim3*, and *six3* indicate that *yot* mutations disrupt the formation of the rostral portion of the anterior pituitary as well as the adjacent region of the postoptic area. In contrast, markers for optic stalk (*pax2*), hypothalamus (*dlx2*), and telencephalon (*dlx2*, *pax6*) are expressed nor-

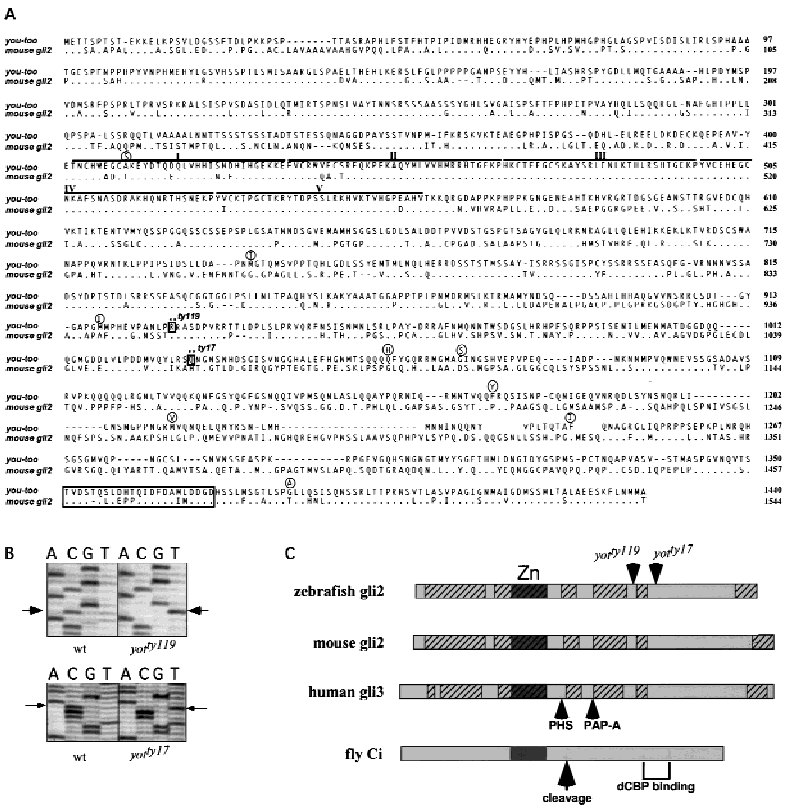

Figure 2. Sequence of zebrafish *gli2* and identification of point mutations in *yot* alleles. (*A*) The deduced amino acid sequence of zebrafish *gli2* (*you-too*) aligned with mouse *gli2*. The five zinc finger regions are indicated by lines and Roman numerals. The positions of stop codons in yot^{ty119} and yot^{ty17} are indicated by boxes and asterisks. Amino acid polymorphisms seen in wild-type strains are shown in circles over the zebrafish *gli2* sequence. A putative VP16-like activation domain at the carboxyl terminus is boxed. (*B*) Sequencing gels showing point mutations in the two *yot* alleles. In yot^{ty119} C3148 is mutated to U, changing Arg-929 to a stop codon. In yot^{ty17} C3445 is mutated to U, changing Glu-1032 to a stop codon. (*C*) Schematic representation of the zebrafish *gli2* protein sequence showing the positions of the stop codons (arrowheads) in the two mutant alleles. Alignment is shown with mouse Gli2, human GLI3, and fly Ci. Hatched regions have >70% sequence identity between the three Gli proteins. Overall amino acid sequence identities are zebrafish *gli2* to mouse *Gli2*, 56%; zebrafish *gli2* to mouse *Gli1*, 29%; zebrafish *gli2* to zebrafish *Gli1*, 35%; zebrafish *gli1* to mouse *Gli1*, 45%; zebrafish *gli1* to mouse *Gli2*, 35% (R.O. Karlstrom, A. Kawakami, W.S. Talbot, A.F. Schier, unpubl.). Sites of stop codons resulting from nonsense mutations in Gli3 that lead to PAP-A and PHS are indicated by arrows. Site of cleavage that results in a repressor form of Ci is shown by an arrow. The carboxy-terminal region of Ci implicated in CBP binding is indicated by brackets. Zinc finger regions (Zn) are indicated by dark boxes.

mally (not shown). These results are consistent with the idea that axon path-finding defects are caused by abnormal development of the postoptic area.

To further examine the role of Gli2 in mediating Hh signals during forebrain development, we analyzed the effect of *yot* on known Hh target genes. The putative Hh receptor *patched-1* (*ptc-1*) is expressed in Hh-responsive cells adjacent to the source of Hh (Fig. 5E,F; Concordet et al. 1996) and has been shown to be up-regulated by Hh signaling in flies and vertebrates (Concordet et al. 1996; Ingham 1998). Consistent with defects in Hh signaling, we find that *ptc-1* expression in *yot* mutant embryos is reduced throughout the brain and expression is absent in the postoptic area (arrow in Fig. 5F). The *pax6* gene has been shown to be negatively regulated by *shh* in zebrafish (Ekker et al. 1995; Macdonald et al. 1995). Consistent with a role of *gli2* in this repression, *pax6* expression is expanded in *yot* mutants (Fig. 5H). The altered expression of *pax6* and *ptc-1* provides further evidence that *yot* disrupts Hh signaling in the forebrain. The relatively mild effects, however, also indicate that some Hh signaling remains in the ventral neural tube of *yot* mutants. It is conceivable that the carboxy-terminal truncations of Gli2 in *yot* mutants do not eliminate all Gli2 activity. In addition, other factors such as Gli1 or Gli3 might mediate Hh signaling in *yot* mutants. In support of this idea, we have found that zebrafish *gli1* is expressed in the ventral neurectoderm of wild-type and *yot* mutant embryos (R.O. Karlstrom, W.S. Talbot, and A.F. Schier, unpubl.).

shh has been implicated in the induction of floor plate and ventral motoneurons (Chiang et al. 1996; Dodd et al. 1998), but the role of *gli2* in this process is less clear. Loss-of-function studies in the mouse demonstrate that *gli2* is essential for floor plate, but not motoneuron, development (Ding et al. 1998; Matise et al. 1998). In contrast, overexpression studies in *Xenopus* have led to the proposal that *gli2* represses floor plate development and mediates induction of motoneurons (Ruiz i Altaba 1998). Yet another result is observed in zebrafish. The Gli2 truncations lead to spinal cord defects but do not interfere with the induction of medial floor plate cells or motoneurons (Fig. 5I,J; Brand et al. 1996; Schauerte et al. 1998). The contrasting mouse and zebrafish mutant phenotypes might be due to differences in alleles, that is, the mouse *gli2* mutation deletes zinc fingers 3–5 and more carboxy-terminal sequences, whereas the zinc finger region is unaffected in the *yot* mutations. As mentioned above, other factors that mediate Hh signaling in zebrafish, including *gli1*, might compensate for the mutations in *gli2*. It is interesting to note, however, that like *yot*, null mutations in zebrafish *shh* do not affect medial floor plate development (Schauerte et al. 1998). Thus, in contrast to mouse, medial floor-plate formation in zebrafish might be independent of Hh signaling (Schauerte et al. 1998). Alternatively, the presence of *echidna hedgehog* and *tiggy-winkle hedgehog*, as well as additional *gli* genes, might compensate for mutations in zebrafish *shh* and *gli2*, respectively (Dodd et al. 1998). The isolation of mutations in other *gli* and *hh* genes will be an important step toward determining the exact role

of *gli* genes in mediating Hh signaling in zebrafish and will allow a more careful comparison of the developmental role of this signaling pathway in different vertebrates.

In summary, using comparative synteny in zebrafish for the first time, we have found that *yot* mutations encode carboxy-terminally truncated Gli2 proteins that affect Hh signaling. The use of synteny conservation, in combination with the rapid development of genomic resources in human, mouse, and zebrafish, is likely to facilitate the cloning of zebrafish mutations. Although ectopic expression experiments suggested a role for *gli* genes in ventral CNS specification (Hynes et al. 1997; Lee et al. 1997; Ruiz i Altaba 1998), it was previously unknown whether mutations in this gene family would affect ventral forebrain development or retinal axon pathfinding. The defects in *yot* mutant embryos underscore the importance of *gli* genes and the Hh signaling pathway in specifying cell fates in the ventral forebrain. We propose that these defects affect cellular cues that guide axons across the midline of the ventral forebrain and disrupt postoptic commissure formation and optic nerve crossing. Our phenotypic analysis also suggests a role for Hh signaling in differentiation of the anterior pituitary and inhibition of lens induction in this region. The defects in *yot* mutants and the involvement of *shh* in human holoprosencephaly (for review, see Ming and Muenke 1998) suggest that mutations in *gli2* might underlie human diseases such as congenital malformations in facial and forebrain midline structures.

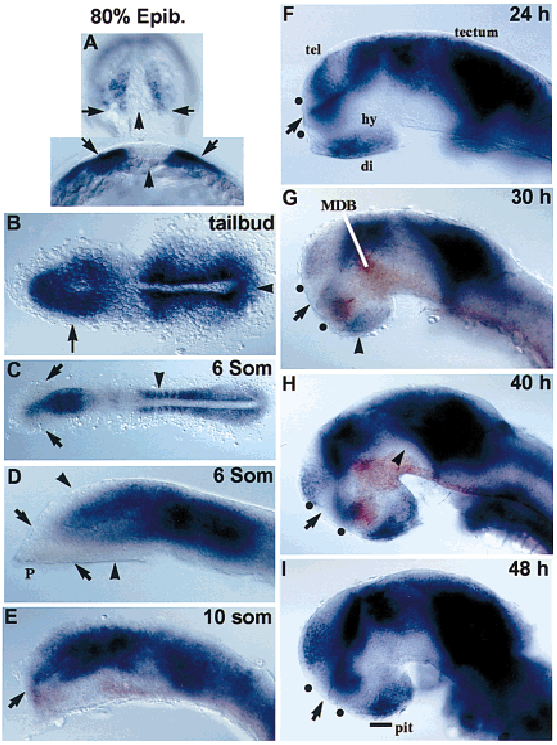

Figure 3. Zebrafish *gli2* RNA expression (purple). Embryos in *E*, *G*, and *H* were double labeled with *shh* (brown). Anterior is to the left in all panels except *A*. Dots in *F–I* show positions of anterior and postoptic commissures. (*A*) 80% epiboly. (*Top*) Dorsal view, animal pole up, arrows point to the vegetal margin of the gastrula. (*Bottom*) Optical cross section, dorsal up, arrowhead marks midline. (Arrows) Lateral mesoderm. (*B*) Tailbud stage, dorsal view. (Arrow) Anterior neural plate; (arrowhead) tailbud. (*C*) Six-somite stage, dorsal view. (Arrows) Optic vesicles; (arrowhead) developing somites. (*D–I*) Lateral views of the brain, medial focal plane, eyes have been removed. (*D*) Six-somite stage. (Arrowheads) Most dorsal telencephalon and ventral diencephalon do not express *gli2;* (P) mesendodermal tissue of the polster. (Arrows) Border between polster and forebrain tissue. (*E*) 10-somite stage. (Arrow) Optic recess. (*F*) 24 hr; (*G*) 30 hr; (*H*) 40 hr. Expression is reduced in cells adjacent to *shh*-expressing cells in the forebrain (arrowhead). (*I*) 48 hr. Line, rostral portion of the anterior pituitary anlage. (di) Diencephalon; (hy) hypothalamus; (MDB) mid-diencephalon boundary, (pit) anterior pituitary; (tel) telencephalon; (dots) anterior and postoptic commissures.

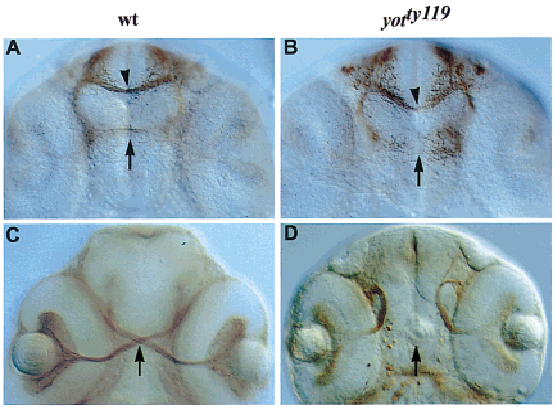

Figure 4. *yot* affects axon guidance in the ventral forebrain. (*A–D*) Ventral view, anterior up. (*A*,*B*) Anti-acetylated tubulin labeling. (*A*) In 36-hr wild-type embryos, commissural axons have crossed the forebrain to form the anterior (arrowhead) and postoptic (arrow) commissures. (*B*) In *yot* mutant embryos, the postoptic commissure fails to form (arrow) and the anterior commissure appears reduced (arrowhead). (*C*,*D*) ZN-8 antibody labeling. (*C*) In 48-hr embryos, retinal axons have crossed the midline to form the optic chiasm (arrow). (*D*) In *yot* mutant embryos, axons project ipsilaterally after leaving the eye and no chiasm forms. Lens-like structures form in *yot*^{ty119} mutant embryos in the ventral diencephalon (arrow).

Materials and methods

Mutant and mapping strains

Two alleles of *yot* (*yot*^{ty17} and *yot*^{ty119}) were identified previously in mutant screens (Brand et al. 1996; Karlstrom et al. 1996; van Eeden et al. 1996). Both alleles were kept in the Tübingen background and were also outcrossed to the AB background. For mapping, *yot*^{ty17} was crossed to two polymorphic lines, the WIK line (Rauch et al. 1997) and the Leopard/Long Fin line. In situ and antibody analyses were performed with both the stronger (*yot*^{ty119}) and weaker (*yot*^{ty17}) alleles.

Genetic mapping by centromere linkage analysis

We determined the position of *yot* on the zebrafish genetic map using centromere linkage analysis (Johnson et al. 1996; Postlethwait and Talbot 1997). Gynogenetic diploid embryos were obtained from heterozygous females by early pressure treatment of eggs fertilized with inactivated sperm. Mutant and wild-type progeny were identified by visual inspection on day 1 or day 2. DNA prepared from individuals or from pools of eight mutant or wild-type individuals was assayed by PCR using polymorphic markers (simple sequence length polymorphisms; Knapik

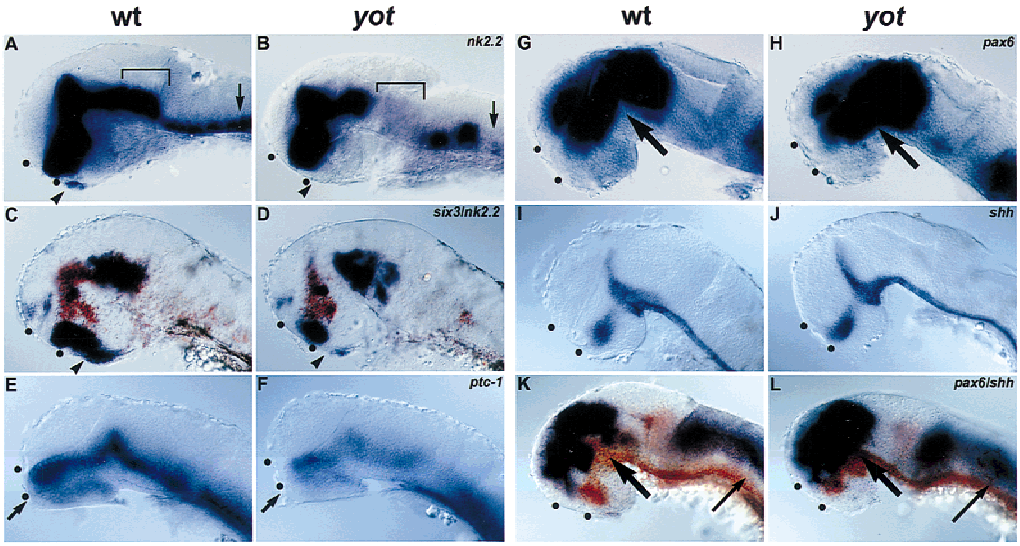

Figure 5. *yot* affects anterior pituitary formation and expression of Hh target genes in the brain. Expression of *nk2.2* (*A,B*), *six3* (C,D), *ptc-1* (*E,F*), *pax6* (*G,H,K,L*), and *shh* (*I,J*) in purple. Embryos in *C*, *D*, *K*, and *L* were double labeled with *nk2.2* (*C,D*) or *shh* (*K,L*) (brown). Anterior is to the *left* in all panels; eyes have been removed. (*A–D*) 36 hr. (*A,B*) (Arrowheads) Anterior pituitary anlage; (square brackets) regions of the tegmentum; (arrows) ventral hindbrain. (*C,D*) In *yot* mutants, *six3* expression (purple) is absent from the middle of its expression domain, which includes the rostral part of the anterior pituitary anlage (arrowheads). (*E,F*) 30 hr. (Arrow) Postoptic area. (*G–L*) 36 hr. Expansion of *pax6* expression at mid-diencephalic boundary (thick arrows) and dorsal-to-*shh*–expressing cells (thin arrows). (*K,L*) (Dots) Anterior and postoptic commissures.

et al. 1998). This identified two genetic markers (z5080 and z3124) on LG9 that were linked to *yot*. Finer mapping was done using embryos obtained from pairwise matings of heterozygous *yot* parents in a WIK background.

Cloning zebrafish gli *genes*

Genomic clones were obtained by screening a gridded genomic bacterial artificial chromosome (BAC) library (Genome Systems) using radiolabeled probes for a mouse *gli2* cDNA at low stringency hybridization conditions. BAC DNA was prepared for positive clones and the BAC ends were sequenced using vector primers. Sequence from the end of clone 100D2 had homology to *gli2*. Sequence from this clone was used to design primers that amplified a single strand conformation polymorphism (SSCP), which was used to map the BAC end to LG9 and to test cosegregation with the *yot* locus (Talbot and Schier 1999).

cDNA clones encoding *gli* genes were isolated from a 15- to 19-hr embryonic cDNA library (generously provided by Bruce Appel and Judith Eisen, University of Oregon, Eugene). BAC 100D2 sequence was used to design PCR primers that were used to make radiolabeled PCR probes. A low stringency screen using the mouse *gli2* cDNA was also performed. Three clones encoding portions of the *gli2* gene were identified. These clones were sequenced and their sequences assembled into the full *gli2* coding region (GenBank accession no. AF085746) containing a putative translation initiation codon at position 364 and a stop codon at position 4681.

Sequencing mutant alleles

RT–PCR and cycle sequencing were used to sequence the two ENU-induced *yot* alleles (Talbot and Schier 1999). RNA was isolated from the following pools of 40 embryos: (1) yot^{ty119} wild-type siblings; (2) yot^{ty119} mutants; (3) yot^{ty17} wild-type siblings; and (4) yot^{ty17} mutants. First-strand cDNA was made using Superscript reverse transcriptase (GIBCO). Fragments (500–1000 bp) were amplified from first strand cDNA by the PCR using primers based on the deduced *gli2* cDNA sequence. DNA fragments were then gel purified and cycle sequenced (Stratagene Cyclist). Sequence was compared between pools and to the *gli2* cDNA sequence. The fragments containing the *yot* point mutations were also subcloned using the TA cloning system (Invitrogen). DNA from two separately isolated clones was purified and the mutant sequence was verified.

In situ hybridization and antibody labeling

Single-color in situ labeling was performed as described (Schier et al. 1997). Antibody labeling (Karlstrom et al. 1996) was performed using the anti-acetylated tubulin antibody (Sigma) to label axon tracts and ZN-8 to label retinal ganglion cells and their axons. Two-color in situ labeling (Jowett and Yan 1996) was performed using the INT substrate (Boehringer Mannheim) for the second phosphatase reaction. A 3′ *gli2* probe lacking the conserved zinc finger region was made using pBS–gli2-4.9 linearized with *Bst*98-1 and T7 RNA polymerase. Other probes used were *nk2.2* (Barth and Wilson 1995), *lim3* (Glasgow et al. 1997), *shh* (Krauss et al. 1993), *ptc-1* (Concordet et al. 1996), and *pax6* (Krauss et al. 1991). Our *six3* probe corresponds to that of one group (Kobayashi et al. 1998) but is called *six6* by another (Seo et al. 1998).

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References

Akimaru, H., Y. Chen, P. Dai, D.X. Hou, M. Nonaka, S.M. Smolik, S. Armstrong, R.H. Goodman, and S. Ishii. 1997. Drosophila CBP is a co-activator of cubitus interruptus in hedgehog signaling. *Nature* **386:** 735–738.

Alexandre, C., A. Jacinto, and P.W. Ingham. 1996. Transcriptional activation of *hedgehog* target genes in *Drosophila* is mediated directly by the Cubitus interruptus protein, a member of the GLI family of zinc finger DNA-binding proteins. *Genes & Dev.* **10:** 2003–2013.

Amores, A., A. Force, Y.-L. Yan, L. Joly, C. Amemiya, A. Fritz, R. Ho, J. Langeland, V. Prince, Y.-L. Wang, M. Westerfield, M. Ekker, and J. Postlethwait. 1998. Zebrafish *hox* clusters and vertebrate genome evolution. *Science* **282:** 1711–1714.

Aza-Blanc, P., F.-A. Ramirez-Weber, M.-P. Laget, C. Schwartz, and T.B. Kornberg. 1997. Proteolysis that is inhibited by hedgehog targets Cubitus interruptus protein to the nucleus and converts it to a repressor. *Cell* **89:** 1043–1053.

Barth, K.A. and S.W. Wilson. 1995. Expression of zebrafish nk2.2 is influenced by sonic hedgehog/vertebrate hedgehog-1 and demarcates a zone of neuronal differentiation in the embryonic forebrain. *Development* **121:** 1755–1768.

Brand, M., C.-P. Heisenberg, R. Warga, F. Pelegri, R. Karlstrom, D. Beuchle, A. Picker, Y.-J. Jiang, M. Furutani-Seiki, F. van Eeden, M. Granato, P. Haffter, M. Hammerschmidt, D. Kane, R. Kelsh, M. Mullins, J. Odenthal, and C. Nüsslein-Volhard. 1996. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. *Development* **123:** 129–142.

Chiang, C., Y. Litingtung, E. Lee, K.E. Young, J.L. Corden, H. Westphal, and P.A. Beachy. 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. *Nature* **383:** 407–413.

Concordet, J.P., K.E. Lewis, J.W. Moore, L.V. Goodrich, R.L. Johnson, M.P. Scott, and P.W. Ingham. 1996. Spatial regulation of a zebrafish patched homologue reflects the roles of sonic hedgehog and protein kinase A in neural tube and somite patterning. *Development* **122:** 2835–2846.

Ding, Q., J. Motoyama, S. Gasca, M. Rong, H. Sasaki, J. Rossant, and C.-C. Hui. 1998. Diminished Sonic hedgehog signaling and lack of floor plate differentiation in gli2 mutant mice. *Development* **125:** 2533–2543.

Dodd, J., T. Jessell, and M. Placzek. 1998. The when and where of floor plate induction. *Science* **282:** 1654–1657.

Ekker, S.C., A.R. Ungar, P. Greenstein, D.P. vonKessler, J.A. Porter, R.T. Moon, and P.A. Beachy. 1995. Patterning activities of vertebrate hedgehog proteins in the developing eye and brain. *Curr. Biol.* **5:** 944–955.

Glasgow, E., A.A. Karavanov, and I.B. Dawid. 1997. Neuronal and neuroendocrine expression of lim3, a LIM class homeobox gene, is altered in mutant zebrafish with axial signaling defects. *Dev. Biol.* **192:** 405–419.

Hammerschmidt, M., A. Brook, and A.P. McMahon. 1997. The world according to hedgehog. *Trends Genet.* **13:** 14–21.

Hynes, M., D.M. Stone, M. Dowd, S. Pitts-Meek, A. Goddard, A. Gurney, and A. Rosenthal. 1997. Control of cell pattern in the neural tube by the zinc finger transcription factor and oncogene gli-1. *Neuron* **19:** 15–26.

Ingham, P.W. 1998. Transducing Hedgehog: The story so far. *EMBO J.* **17:** 3505–3511.

Johnson, S.L., M.A. Gates, M. Johnson, W.S. Talbot, S. Horne, K. Baik, S. Rude, J.R. Wong, and J.H. Postlethwait. 1996. Centromere-linkage analysis and consolidation of the zebrafish genetic map. *Genetics* **142:** 1277–1288.

Jowett, T. and Y.-L. Yan. 1996. Two colour whole-mount in situ hybridisation. *Trends Genet.* **12:** 387–389.

Kang, S., J.M.J. Graham, A.H. Olney, and L.G. Biesecker. 1997. GLI3 frameshift mutations cause autosomal dominant Pallister-Hall syndrome. *Nat. Genet.* **15:** 266–268.

Karlstrom, R., T. Trowe, S. Klostermann, H. Baier, M. Brand, A. Crawford, B. Grunewald, P. Haffter, H. Hoffman, S.U. Meyer, B. Müller, S. Richter, F. van Eeden, C. Nüsslein-Volhard, and F. Bonhoeffer. 1996. Zebrafish mutations affecting retinotectal axon pathfinding. *Development* **123:** 427–438.

Knapik, E.W., A. Goodman, M. Ekker, M. Chevrette, J. Delgado, S. Neuhauss, N. Shimoda, W. Driever, M.C. Fishman, and H.J. Jacob. 1998. A microsatellite genetic linkage map for zebrafish (*Danio rerio*). *Nat. Genet.* **18:** 338–343.

Kobayashi, M., R. Toyama, H. Takeda, I.B. Dawid, and K. Kawakami. 1998. Overexpression of the forebrain-specific homeobox gene six3 induces rostral forebrain enlargement in zebrafish. *Development* **125:** 2973–2982.

Krauss, S., T. Johansen, V. Korzh, and A. Fjose. 1991. Expression of the zebrafish pax genes suggests a role in early brain regionalization. *Nature* **353:** 267–270.

Krauss, S., J.-P. Concordet, and P.H. Ingham. 1993. A functionally conserved homolog of the Drosophila segment polarity gene hh is expressed in tissues with polarizing activity in zebrafish embryos. *Cell* **75:** 1431–1444.

Lee, J., K.A. Platt, P. Censullo, and A. Ruiz i Altaba. 1997. gli1 is a target of sonic hedgehog that induces ventral neural tube development. *Development* **124:** 2537–2552.

Macdonald, R., K.A. Barth, Q. Xu, N. Holder, I. Mikkola, and S.W. Wilson. 1995. Midline signaling is required for Pax gene regulation and patterning of the eyes. *Development* **121:** 3267–3278.

Matise, M., D. Epstein, H. Park, K. Platt, and A. Joyner. 1998. gli2 is required for the induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. *Development* **125:** 2759–2770.

Ming, J.E. and M. Muenke. 1998. Holoprosencephaly: From Homer to Hedgehog. *Clin. Genet.* **53:** 155–163.

Placzek, M. 1995. The role of the notochord and floor plate in inductive interactions. *Curr. Opin. Genet. Dev.* **5:** 499–506.

Postlethwait, J.H. and W.S. Talbot. 1997. Zebrafish genomics: From mutants to genes. *Trends Genet.* **13:** 183–190.

Postlethwait, J.H., Y.L. Yan, M.A Gates, S. Horne, A. Amores, A. Brownlie, A. Donovan, E.S. Egan, A. Force, Z. Gong, C. Goutel, A. Fritz, R. Kelsh, E. Knapik, E. Liao, B. Paw, D. Ransom, A. Singer, M. Thomson, T.S. Abduljabbar, P. Yelick, D. Beier, J.S. Joly, D. Larhammar, and W.S. Talbot. 1998. Vertebrate genome evolution and the zebrafish gene map. *Nat. Genet.* **18:** 345–349.

Radhakrishna, U., A. Wild, K.-H. Grzeschik, and S.E. Antonarakis. 1997. Mutation in Gli3 in postaxial polydactyly type A. *Nat. Genet.* **17:** 269–271.

Rauch, G.J., M. Granato, and P. Haffter. 1997. A polymorphic zebrafish line for genetic mapping using SSLPs on high percentage agarose gels. *Trends Tech. Tips Online*. TO1208

Ruiz i Altaba, A. 1997. Catching a Gli-mpse of hedgehog. *Cell* **90:** 193–196.

———. 1998. Combinatorial Gli function in floor plate and neuronal inductions by Sonic hedgehog. *Development* **125:** 2203–2212.

Schauerte, H., F. van Eeden, C. Fricke, J. Odenthal, U. Strähle, and P. Haffter. 1998. Sonic hedgehog is not required for the induction of medial floor plate cells in zebrafish. *Development* **125:** 2983–2993.

Schier, A.F., S.C.F. Neuhauss, K.A. Helde, W.S. Talbot, and W. Driever. 1997. The one-eyed pinhead gene functions in mesoderm and endoderm formation in zebrafish and interacts with no tail. *Development* **124:** 327–342.

Seo, H.C., O. Drivenes, S. Ellingsen, and A. Fjose. 1998. Expression of two zebrafish homologues of the murine Six3 gene demarcates the initial eye primordia. *Mech. Dev.* **73:** 45–57.

Talbot, W.S. and A.F. Schier. 1999. Positional cloning of mutated genes in zebrafish. *Methods Cell Biol.* **60:** 259–286.

van Eeden, F., M. Granato, U. Schach, M. Brand, M. Furutani-Seiki, P. Haffter, M. Hammerschmidt, C.-P. Heisenberg, Y.-J. Jiang, D. Kane, R. Kelsh, M. Mullins, J. Odenthal, R. Warga, M. Allende, E. Weinberg, and C. Nüsslein-Volhard. 1996. Mutations affecting somite formation and patterning in the zebrafish, *Danio rerio*. *Development* **123:** 153–164.

Yoon, J., C. Liu, J. Yang, R. Swart, P. Iannaccone, and D. Walterhouse. 1998. GLI activates transcription through a herpes simplex viral protein 16-like activation domain. *J. Biol. Chem.* **273:** 3496–3501.