The zebrafish iguana locus encodes Dzip1, a novel zinc finger protein required for proper regulation of hedgehog signaling

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Introduction

During animal development, cell fates are specified in response to gradients of signaling molecules within the embryo. Members of the Hedgehog (Hh) family of intercellular signaling molecules control a variety of developmental processes, ranging from segment patterning in Drosophila to organogenesis, left-right asymmetry and dorsoventral patterning of the spinal cord and forebrain in vertebrates (reviewed by Ingham and McMahon, 2001; McMahon et al., 2003). Aberrant regulation of Hh signaling in humans causes developmental defects such as holoprosencephaly (HPE) (reviewed by Wallis and Muenke, 2000) and postaxial polydactyly (Radhakrishna et al., 1997), and can also lead to various types of cancers, including basal cell carcinoma and medulloblastoma (reviewed by Goodrich and Scott, 1998; Ruiz i Altaba, 1999b; Villavicencio et al., 2000).

Because Hh signaling plays such a central role in development and disease, the Hh signaling pathway has been investigated in considerable detail. Genetic and in vitro studies in Drosophila have revealed that Hh signals are transduced by binding of Hh ligands to the Patched (Ptc) cell-surface receptor, resulting in the activation of the transmembrane protein Smoothened (Smo). In Drosophila, the intracellular regulation of Hh signaling is mediated by post-translational modifications of Cubitus interruptus (Ci), a zinc-finger-containing transcription factor of the Gli family that can be both an activator and a repressor of Hh target genes (Methot and Basler, 2001). In the absence of Hh signal, proteolytic cleavage converts Ci to a transcriptional repressor (Aza-Blanc et al., 1997; Wang and Holmgren, 1999). In the presence of Hh signals, cleavage of Ci is inhibited and a full-length activator isoform predominates. In vertebrates, at least three Gli genes,
Gli1, Gli2 and Gli3, mediate the transcriptional response to Hh signals (Hui et al., 1994; Ruiz i Altaba, 1998). The functions of these different Gli genes have been analyzed in mouse, Xenopus, zebrafish and cultured cells (reviewed by Ingham and McMahon, 2001; Koebenrick and Pieler, 2002; Ruiz i Altaba et al., 2002). Although Gli1 is dispensable for normal mouse development (Park et al., 2000; Bai et al., 2002), analysis of Gli1+/–; Gli2–/– mutants (Park et al., 2000) and the rescue of Gli2 mutant by Gli1 (Bai and Joyner, 2001) suggest that Gli1 has an activator function. Consistently, gli1 mutations in zebrafish lead to a loss of Hh target gene expression (Karlstrom et al., 2003). As yet there is no evidence that Gli1 activity is regulated by protein processing, it appears to be solely an activator of the Hh response (Epstein et al., 1996; Marigo et al., 1996; Hynes et al., 1997; Lee et al., 1997; Dai et al., 1999).

By contrast, Gli2 appears to be both an activator and repressor of Hh target genes, depending on the tissue being examined. Mouse Gli2 mutations are perinatal lethal and result in the downregulation of Hh target genes (Ding et al., 1998; Matise et al., 1998). The C-terminal region of Gli2 appears to be essential for the activation function, as the C-terminally truncated Gli2 proteins do not activate Hh target genes (Karlstrom et al., 1999; Ruiz i Altaba, 1999a; Sasaki et al., 1999; Karlstrom et al., 2003). Several studies have shown that Gli2 can also repress the expression of Hh target genes (von Mering and Basler, 1999; Sasaki et al., 1999; Aza-Blanc et al., 2000; Karlstrom et al., 2003). Similarly, several lines of evidence suggest that Gli3 can act both as an activator and as a repressor of Hh signaling (Masuya et al., 1995; Dai et al., 1999; Ruiz i Altaba, 1999a; Sasaki et al., 1999; Shin et al., 1999; Aza-Blanc et al., 2000; Tole et al., 2000; Litingtung and Rubin, 2000). The transport of Ci to the nucleus is mediated by another protein, Suppressor of fused (Su(Fu)). In addition to such a positive regulation in vertebrates, Sonic hedgehog (Shh) is expressed in the notochord and floor plate (Echelard et al., 1993; Krauss et al., 1993; Roelink et al., 1994; Ekker et al., 1995), and is essential for the induction of floor plate, motoneurons and a class of ventral interneurons in the neural tube (Chiang et al., 1996; Ericson et al., 1996). Shh signaling is also required for the induction of muscle and sclerotome cell types in somites (reviewed by Bumcrot and McMahon, 1995). A large number of zebrafish mutations, collectively called the midline mutants, have been identified that lead to ventral neural tube defects, absence of an optic chiasm and defects in slow muscle fiber formation (Brand et al., 1996; Chen et al., 1996; Karlstrom et al., 1996; van Eeden et al., 1996b). Many of these midline mutants have now been shown to encode components of the Hh signal cascade. shh is disrupted in sonic-you (syu) mutants (Schauerte et al., 1998), smo is disrupted in slow-muscle-omitted (smu) (Chen et al., 2001; Varga et al., 2001), gli2 is disrupted in you-too (yot) (Karlstrom et al., 1999), gli1 is disrupted in detour (dtr) (Karlstrom et al., 2003) and dispatched1 is disrupted in chameleon (con) (Nakano et al., 2004). As several molecularly uncharacterized midline mutants share many phenotypes with these known Hh pathway mutants, it is likely that they encode additional components of Hh signaling.

In this study, we show that igu mutations lead to reduced Hh target gene expression in the neural tube. Surprisingly, these same mutations cause the ectopic activation of Hh target genes in somites. Our analyses reveal that IgU function is required for the full activation of Hh signaling in response to Hh ligands. We also show that the igu mutations, directly or indirectly, affect the negative regulation of Hh signaling that is required for silencing the Hh target gene expression in the absence of Hh ligands. Positional cloning of the igu gene revealed that the gene encodes Dzip1, a novel component of the Hh signaling pathway. We show that Igu/Dzip1 acts as a necessary permissive factor for the proper regulation of Hh signaling.

Materials and methods
Fish strains and genetics
The following zebrafish mutants were used, syu (thx392), yot (ty119), dtr (ts269), con (tm15a) and igu (ms97a and ts294e). Two alleles of igu mutants were indistinguishable in all phenotypes, including marker gene expression. Therefore, we used igu(ts294e) embryos for most of our analyses. For fine mapping, the DNA markers of simple sequence length polymorphisms (SSLP) (Knapik et al., 1998) and expressed sequence tag (EST) in the vicinity of igu locus on linkage group 6 (LG6) (http://zfin.org/) were selected for scoring meiotic recombination in igu(ts294e) mutation (Postlethwait and Talbot, 1997). Among 23 DNA markers that we used, the sox21 marker showed one recombination event and the EST marker unp172 showed no recombination in 422 meioses (see Fig. 6A). Searches of the GenBank sequence database using the Blast program (http://www.ncbi.nlm.nih.gov/blast/) identified overlapping PAC clones that contained unp172, 26624 and sox21. In the igu region, three genes, claudin10, a component of tight junctions, ATP-binding
cassette transporter 4 (abcc4) and dzip1, were predicted by using the GenScan program (http://genes.mit.edu/GENSCAN.html).

Sequence analysis and characterization of igu mutation

To identify the genetic lesions associated with the two igu alleles, the dzip1-coding regions was amplified by PCR from reverse-transcribed RNAs made from wild-type and mutant embryos at 30 hours post fertilization (hpf), and these PCR products were directly sequenced. The point mutations were also confirmed by sequencing subcloned cDNAs and genomic DNAs. The sequences of dzip1 cDNAs were deposited in GenBank under Accession Numbers AB106357 and AB106358.

RNA injection

For RNA injections, PCR fragments of igu-coding sequences from wild-type and mutant alleles were cloned into pCS2+ vector. Capped mRNAs were synthesized in vitro using the mMessage Machine kit (Ambion) according to the manufacturer’s instructions and injected into embryos (1- to 4-cell stage) that were obtained from igu<sup>+</sup>-incrosses.

Cyclopamine and forskolin treatments

Cyclopamine and forskolin were dissolved in 100% dimethyl sulfoxide (DMSO) at 10 mM and 60.9 mM, respectively. Embryos were dechorionated with pronase at 50% epiboly and placed in 6 ml of embryo medium (Westerfield, 1993) containing 50 mM KC1, 0.3% Tween 20, 0.3% NP40, 1 mM EDTA) for 5 hours to overnight at 55°C. For genotyping, 500 mM KC1, 0.3% Tween 20, 0.3% NP40, 1 mM EDTA) for 5 hours in 80% glycerol.

In situ hybridization and genotyping

Whole-mount in situ hybridization was performed as described (Schier et al., 1997). Embryos were photographed in 80% glycerol, and genomic DNA was recovered from each embryo by proteinase K treatment (1 mg/ml) in 50 μl of lysis buffer (10 mM Tris-HCl pH 8, 50 mM KC1, 0.3% Tween 20, 0.3% NP40, 1 mM EDTA) for 5 hours to overnight at 55°C. For genotyping, 5 μl of genomic DNA was used for each PCR reaction. Mutant embryos were identified using tightly linked genetic markers. Primers used for genotyping were unp172F (5'-TCATGACGAAGCAGTTTGGA-3') and unp172R (5'-CAGGTGGTCCAGGTTA-3') for igu<sup>6294c</sup>, z1660 for yot<sup>119</sup>, and z14475 for dtr<sup>ps269</sup>.

Transfection and antibody staining

PCR amplified wild-type and mutant (ts<sup>294e</sup> allele) igu/dzip1-coding sequences were subcloned into pcDNA4-HisMax TOPO (Invitrogen). Transfection into NIH3T3 or HEK293T cells was performed according to the manufacturer’s instructions (Fugene, Roche). Cells were fixed at 2 days, permeabilized with methanol and stained with Omni-probe antibody (Santa Cruz Biotechnology) in combination with anti-rabbit FITC (Jackson ImmunoResearch). Lysosomes were visualized with anti-Lamp1 antibody (Hughes and August, 1981) in NIH3T3 cells. We also made FLAG-tagged constructs and obtained a similar result (data not shown).

Results

Aberrant regulation of Hh signaling in igu mutants

Zebrafish midline mutants have a characteristic curved body shape and defects in cell fate specification in the ventral neural tube and somites. Careful analyses of the different midline mutants have revealed a range of phenotypes that help uncover the differential contributions of various Hh pathway components to overall Hh signal transduction (Brand et al., 1996; Chen et al., 1996; van Eeden et al., 1996a; van Eeden et al., 1996b; Karlstrom et al., 1996; Lewis et al., 1999; Odenthal et al., 2000; Sbroga et al., 2003). To examine more carefully the regional regulation of Hh signaling, we examined the expression of Hh target genes in five midline mutants, syu/shh, dtr/gli1, yot/gli2, con and igu. In the neural tube, the expression of nk2.2, a Hh-induced marker for ventral neuroectoderm (Ericson et al., 1996), is variably reduced in all mutants (Fig. 1A1-F1). However, markers for adjacent dorsal regions, such as pax2 and lim5, are largely unaffected (data not shown). Based on defects in nk2.2 and pax6 expression, these mutants can be ranked in order of severity: con<sup>+</sup>yot/gli2<sup>2</sup>dtr/gli1<sup>+</sup> syu/shh<sup>+</sup>iguyot/gli2<sup>+</sup> con. Thus, nk2.2 expression defects are least severe in igu mutants, with punctate nk2.2 expression remaining in the trunk region (Fig. 1B1). nk2.2 expression defects are most severe in con and yot/gli2 (Fig. 1E1,F1). Consistently, the expression of pax6, a gene that is expressed in dorsally adjacent regions of the neural tube and negatively regulated by Hh signaling (Ékker et al., 1995; Macdonald et al., 1995; Barth and Wilson, 1995; Ericson et al., 1997), is expanded ventrally in yot/gli2 and con mutants, but not in igu or other mutants (Fig. 1A3-F3). We conclude that defects in Hh signaling are more severe in yot/gli2 and con mutants than in the other mutants examined, while igu mutations impair Hh signaling to a lesser degree. Intriguingly, in dtr/gli1, syu/shh and igu mutants, cells in the ventral spinal cord do not express both nk2.2 and pax6, suggesting that the induction of nk2.2 expression and inhibition of pax6 expression are independently regulated by different threshold responses to Hh signals.

Hh signaling is also required for cell fate determination in somitic mesoderm and is responsible for the induction of muscle pioneer cells (MPCs) and a population of fast fibers around the MPCs termed medial fast fibers (MFFs) (Currie and Ingham, 1996; Hammerschmidt et al., 1996; Blagden et al., 1997; Roy et al., 2001; Wolff et al., 2003). These cell types are marked by strong and weak expression of engrailed1 (en1), respectively (Fig. 1A1,A5). In accordance with the defects of nk2.2 expression in the neural tube, en1 expression in somites is completely lost in con mutants (Fig. 1F4,F5) and severely reduced in syu/shh and yot/gli2 mutants (Fig. 1C4,C5,E4,E5). As previously shown using an anti-En antibody (Wolff et al., 2003), dtr/gli1 mutations do not affect en1 expression in MPCs and MFFs (Fig. 1D4,D5). In striking contrast to the defects seen in the other Hh pathway mutants, igu mutations lead to an increase in en1 positive cells in somites. This suggests either that Hh signaling is increased in igu mutants, or that Hh signals propagate farther from the source of Hh.

To verify that defects in nk2.2 and en1 expression are correlated with the changes in Hh signaling, we examined the expression of patched1 (ptc1), a sensitive indicator of Hh signaling (Concordet et al., 1996; Goodrich et al., 1996). In agreement with the observed en1 phenotypes, ptc1 expression is reduced in somites of syu/shh and yot/gli2 mutants (Fig. 1C4,C5,E4,E5). As previously shown using an anti-En antibody (Wolff et al., 2003), dtr/gli1 mutations do not affect en1 expression in MPCs and MFFs (Fig. 1D4,D5). In striking contrast to the defects seen in the other Hh pathway mutants, igu mutations lead to an increase in en1 positive cells in somites. This suggests either that Hh signaling is increased in igu mutants, or that Hh signals propagate farther from the source of Hh.
expression in somites (Fig. 1B4-B7), indicating that Hh signaling is ectopically activated in somites. The ectopic ptc1 expression is evident as early as 12 hpf (Fig. 2I). By contrast, ptc1 expression in the ventral neural tube appears to be unchanged or slightly decreased compared with wild-type embryos (Fig. 1A7,B7). Thus, igu mutants show ectopic activation of Hh target genes in somites despite the reduction of the Hh target genes ptc1 and nk2.2 in the neural tube. Such an aberrant regulation of Hh target gene expression suggests that the igu gene product may play an important role in both the positive and negative regulation of Hh signaling in different tissues.

**Hh-independent expression of Hh target genes in igu mutants**

To further characterize the role of the igu gene in Hh signaling, we performed epistasis analyses using cyclopamine, an alkaloid that binds and inhibits the function of Smo (Incardona et al., 1998; Taipale et al., 2000). Cyclopamine has been shown to effectively and specifically block Hh signaling in zebrafish (Barresi et al., 2000; Chen et al., 2001; Sbrogna et al., 2003). Embryos treated with cyclopamine lose the expression of Hh target genes (Fig. 2F-J, Table 1) and resemble smu/smo loss-of-function mutants (Chen et al., 2001; Varga et al., 2001). Residual faint expression of ptc1 in the neural tube (Fig. 2I) appears to be independent of Smo, as similar weak ptc1 expression is seen in the neural tube of smu/smo mutants (data not shown). In contrast to wild-type embryos, cyclopamine treatment of igu mutants did not alter the ectopic expression of ptc1 and en1 in somites (Fig. 2K,M,N; Table 1). Surprisingly, seemingly normal expression of myod (Weinberg et al., 1996) in adaxial cells of igu mutants is also resistant to cyclopamine treatment (Fig. 2B,L). Importantly, cyclopamine treatment did not eliminate residual expression of nk2.2 in igu mutants (Fig. 2J). These results show that the activation of Hh target genes in igu mutants is independent of Hh signals in both somitic and neural tissue. Furthermore, the fact that blocking Smo function had no effect on the igu phenotypes suggests that Igu functions downstream of Smo or in a parallel pathway.

![Fig. 1. Aberrant expression of Hh target genes in igu mutants and comparison with four other Hh signaling mutants. (A1-A7) Wild-type, (B1-B7) igu, (C1-C7) syu/shh, (D1-7) dtr/gli1, (E1-E7) yot/gli2 and (F1-F7) con embryos at 30 hpf. (A1,F1,A2,F2) Expression of nk2.2 in the ventral neural tube is reduced in all mutants. Only igu embryos retain a punctate nk2.2 expression (arrowheads in B1, inset is a higher magnification of boxed area). Lateral views are shown in A1-F1 and the respective cross-sections at the middle of the yolk extension are shown in A2-F2. (A3-F3) Expression of pax6 is ventrally expanded in yot/gli2 (E3) and con (F3), but not in other mutants. (A4-F4,A5-F5) Expression of en1 in somites is shown in lateral views and in cross-sections. en1 expression in MPs and MFFs is completely lost in con (F4, F5). A low level of expression persists in syu/shh (C4) and yot/gli2 (E4), and no defect of en1 expression is seen in dtr/gli1 mutants (D4,D5). In contrast to the other four mutants, en1 expression in somites is upregulated in igu embryos (B4,B5). (A6-F7) Expression of ptc1 is shown in whole mount and in cross-sections. Expression of ptc1 is reduced in syu/shh, dtr/gli1, yot/gli2 and con mutants (C7-F7). Expression is most severely reduced in con mutants, and is mildly reduced in the neural tube of syu/shh, dtr/gli1 and yot/gli2 mutants. Note that ptc1 expression is not maintained in somites of dtr/gli1 embryos, despite the presence of en1 expression, and that the ptc1 expression persists in the sclerotome regions of yot/gli2 (E7). In igu embryos, ptc1 expression is upregulated in the entire somites (B6, B7), whereas the expression in neural tube is unaffected or slightly reduced in the trunk, and slightly more reduced in the brain.]

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Attenuated Gli activating function in \textit{igu} mutants

To test whether the activation of Hh target genes in \textit{igu} mutants depends on the activation of the Hh signaling pathway, as opposed to other signaling pathways, we made compound mutants between \textit{igu} and mutants that affect the Gli transcription factors (Fig. 3). To reduce Gli activator function, we made compound mutants with \textit{dtr/gli1}, which is a loss-of-function allele of the \textit{gli1} transcriptional activator (Karlstrom et al., 2003). In \textit{dtr/gli1} \textit{+/–};\textit{igu} \textit{+/-} compound mutants, spinal cord expression of \textit{nk2.2} is completely absent, similar to the phenotype seen in \textit{dtr/gli1} \textit{–/–} mutants (Fig. 3A,D,G). In the somites, posterior expression of \textit{en1} is expanded in \textit{dtr/gli1} \textit{+/–};\textit{igu} \textit{+/-} embryos, similar to \textit{igu} \textit{+/-} mutants, while the expression is reduced in anterior somites (Fig. 3B,E). In \textit{dtr/gli1} \textit{+/–};\textit{igu} \textit{+/-} homozygous double mutants, \textit{en1} expression is further reduced (Fig. 3H). Similarly, the expansion of somitic \textit{ptc1} expression seen in \textit{igu} mutants is reduced when one or two mutant copies of \textit{dtr/gli1} are added (Fig. 3C,F,I). Taken together, these data indicate that the activation of Hh target genes in \textit{igu} mutants partially depends on Gli1 activator function.

To examine how more severe gli mutations affect the \textit{igu} phenotype, we generated compound mutants with \textit{yot/gli2} mutation. Existing \textit{yot/gli2} alleles encode truncated forms of Gli2 that have no activator function and that act as dominant repressors of Gli1-mediated Hh target gene activation (Karlstrom et al., 2003). Strikingly, introduction of one copy of \textit{yot/gli2} into \textit{igu} mutants suppressed the ectopic expression of Hh target genes in the somites and further reduced expression in the neural tube. The resultant phenotype was similar to or even stronger than that seen in \textit{yot/gli2} \textit{–/–} homozygous mutants (Fig. 3M-O). The \textit{yot/gli2} \textit{–/–} embryos treated with cyclopamine. A broad activation of \textit{ptc1} expression in \textit{igu} mutants at 14 hpf (K) is not affected by cyclopamine. Arrows indicate the adaxial cells. Expression of \textit{myod} in adaxial cells is indistinguishable from normal wild-type embryos, and is not affected by cyclopamine (L). Likewise, \textit{en1} and \textit{ptc1} expression in 30 hpf embryos is not affected by cyclopamine. Despite the defect of \textit{nk2.2} expression in \textit{igu} mutants, the residual expression of \textit{nk2.2} in the neural tube is not eliminated by cyclopamine (O). The genotypes of these embryos were confirmed using tightly linked PCR-based genetic markers after in situ staining.

Table 1. Summary of epistasis analyses

<table>
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<tr>
<th>Injected mRNA</th>
<th>myod (12-14hpf)</th>
<th>ptc1 (12-14hpf)</th>
<th>nk2.2</th>
<th>ptc1</th>
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<tbody>
<tr>
<td>shh (250 pg)</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>15 (25%)</td>
<td>16 (20%)</td>
<td>20 (17%)</td>
<td>20 (33%)</td>
</tr>
<tr>
<td>Down</td>
<td>26 (26%)</td>
<td>29 (26%)</td>
<td>60 (21%)</td>
<td>52 (27%)</td>
</tr>
<tr>
<td>dhPKA (250 pg)</td>
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<tr>
<td>Up</td>
<td>15 (25%)</td>
<td>16 (20%)</td>
<td>20 (17%)</td>
<td>20 (33%)</td>
</tr>
<tr>
<td>Down</td>
<td>26 (26%)</td>
<td>29 (26%)</td>
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<tr>
<th>Inhibitors</th>
<th>myod (12-14hpf)</th>
<th>ptc1 (12-14hpf)</th>
<th>nk2.2</th>
<th>ptc1</th>
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<td>Cyclopamine (50 μM)</td>
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<td>33 (30%)</td>
<td>52 (22%)</td>
<td>62 (26%)</td>
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<tr>
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<td>ND</td>
<td>64 (0%)</td>
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<tr>
<td>(150 μM)</td>
<td>28 (3%)</td>
<td>ND</td>
<td>45 (8%)</td>
<td>38 (13%)</td>
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<td>(75 μM)</td>
<td>18 (25%)</td>
<td>19 (30%)</td>
<td>19 (30%)</td>
<td>22 (7%)</td>
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</tbody>
</table>

Embryos were obtained from \textit{igu}+/+ incrosses. Expected genotypes were 25% \textit{igu}+/–, 50% \textit{igu}–/– and 25% homozygous wild type.

Up, respective Hh target genes are ectopically expressed. These embryos have +/- or +/-\textit{igu} genotypes.

Down, Gene expression is downregulated.

\textit{igu} Hh target genes are expressed in the same pattern in \textit{igu} mutants.

Percentages in parenthesis represent the ratios of embryos with \textit{igu}-type gene expression to the total embryos tested for respective gene expressions. All of these embryos have \textit{igu/igu} genotype. Note that \textit{igu} embryos have a seemingly normal \textit{myod} expression pattern.
Fig. 3. Decreased Gli activator function and/or increased Gli repressor function represses ectopic Hh target gene expression in igu embryos. (A–I) Introduction of dtr/gli1 mutant alleles into the igu genetic background suppressed ectopic Hh target gene expressions in somites. In dtr/gli1<sup>–/–</sup> embryos (A–C), the expression of nk2.2 and ptc1 is reduced, but en1 expression is normal. In dtr/gli1<sup>–/–</sup>;igu<sup>–/–</sup> embryos (D–F), nk2.2 expression is similar to dtr/gli1<sup>–/–</sup> embryos (D), with no nk2.2 expression in the posterior neural tube. By contrast, the expression of en1 (E) and ptc1 (F) are similar to that seen in igu<sup>–/–</sup> mutants; however, en1 expression is reduced in anterior somites. In dtr/gli1<sup>–/–</sup>;igu<sup>–/–</sup> embryos (G–I), the decrease of en1 and ptc1 is more evident (H, I), and the overall phenotype including nk2.2 (G) is stronger than in dtr/gli1<sup>–/–</sup> embryos. Insets show higher magnification views of ptc1 expression in the trunk region. (J–O) A drastic reduction of Hh target gene expression is caused by the introduction of a yot/gli2 mutant allele into the igu genetic background. One copy of yot/gli2 completely suppressed ectopic target gene expression (M–O), and the resulting phenotype is similar to or even stronger than that seen in yot/gli2<sup>–/–</sup> embryos (J–L). An arrowhead marks a spot of nk2.2 expression, which is consistently seen only in yot/gli2<sup>–/–</sup> embryos (J) but not in yot/gli2<sup>–/+;igu</sup> embryos (M). The genotypes of these embryos were confirmed by PCR after in situ staining.

**igu mutations impair both the proper activation and repression of Hh target genes**

Previous studies have established that PKA negatively regulates Hh signaling by promoting the proteolytic processing of Ci/Gli proteins and their conversion to repressor isoforms (Ohlmeyer and Kalderon, 1998; Pan and Rubin, 1995; Lepage et al., 1995; Hammerschmidt et al., 1996). This processing is crucial for silencing target gene expression in the absence of Hh ligands. To explore the possible relationship between Igu and the negative regulation of Hh signaling, we manipulated the PKA activity in igu embryos. First, we inhibited PKA activity by overexpressing the dominant-negative regulatory subunit of PKA (dnPKA). This inhibition of PKA activity in wild-type embryos leads to the ectopic activation of Hh target genes in broad regions of the neural tube and somites (Fig. 4A–C) (Hammerschmidt et al., 1996). However, the expression of dnPKA in igu embryos did not induce expression of Hh-responsive genes (Fig. 4D–F; Table 1). As dnPKA is thought to increase full-length Gli activators by disturbing the processing of Gli proteins into repressor isoforms, our results suggest that igu mutations impair the positive regulation of Gli proteins into a fully active state. This is consistent with results from compound mutants (Fig. 3) and results from shh mRNA injections in which Shh overexpression did not affect the igu mutant phenotype either in somites or in the neural tube (Table 1; data not shown).

To further test the relationship between igu mutations and the negative regulation of Hh signaling, we inhibited Hh signaling using forskolin, which activates adenylate cyclase and increases the activity of PKA (Seamon and Daly, 1981). Treatment of wild-type embryos with forskolin in the range of 75-300 μM completely suppressed Hh target gene expression without producing gross morphological abnormalities (Fig. 5A–D; Table 1). If the igu mutation simply disrupted the positive regulation of Gli proteins, we would predict that forskolin treatment of igu mutants would suppress ectopic Hh target gene expression. Although high doses of forskolin (300 μM) did suppress the expression of Hh target genes in igu mutants (Table 1), a lower dose of forskolin that completely inhibits Hh target gene expression in wild-type embryos (75 μM) did not suppress Hh target gene expression in igu mutants (Fig. 5E–H). This result suggests two possibilities: (1) that the negative regulation of Gli proteins is directly impaired in igu
iguana encodes a novel component in Hh signaling

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Fig. 5. Activation of PKA does not efficiently suppress Hh target gene expression. (A-D) In wild-type embryos, the expression of myod at 14 hpf (A), nk2.2 at 30 hpf (B), en1 at 30 hpf (C) and ptc1 at 30 hpf (D) is repressed by 75 μM forskolin treatment. (E-H) In igu embryos, treatment with 75 μM forskolin did not block Hh target gene expression, with expression patterns similar to those seen in untreated igu mutants. Genotypes were confirmed by PCR after in situ analysis.

mutants; or (2) that Igu mutant proteins have acquired a new function that results in the constitutive production of a low level of Gli activator function and indirectly leads to the impaired negative regulation of Hh signaling. In either case, the ectopic activation of Hh target genes in igu mutants could be explained if igu mutations also affect Gli-mediated repression of Hh target genes in cells more distant from the source of Hh signals.

The zinc-finger protein Dzip1 is disrupted in igu mutants

To further understand how the igu mutation affects Hh signaling, we sought to identify the igu gene. Fine genetic mapping placed igu in a small region on linkage group 6 that contained dzip1 as a possible candidate gene (Fig. 6A). Homologs of dzip1 are present in human, mouse and other vertebrates, but the function of these proteins in development has not yet been characterized. Sequence analysis of dzip1 cDNA amplified from wild-type and igu embryos revealed point mutations leading to changes in the protein sequence at amino acid 371 in igu ts294e and amino acid 454 in igu tm79a (Fig. 6B-D). Both of these mutations introduce premature stop codons and result in the C-terminally truncated Igu/Dzip1
proteins. Sequence analysis of the Igu/Dzip1 protein predicts a single zinc-finger domain, a nuclear localization signal (NLS) and two potential PEST sequences that may serve as signals for rapid protein turnover (Rechsteiner and Rogers, 1996). The N-terminal domain of Igu/Dzip1, including the NLS and zinc-finger domain, has a significant higher homology (51% amino acid identity) to human DZIP1 than does the remainder of the protein. In situ expression analysis showed that igu/dzip1 is expressed throughout the embryo starting at 12 hours (data not shown), consistent with the igu mutation affecting multiple tissue types.

This predicted protein structure suggested that Igu/Dzip1 may localize in the cytoplasm and/or nucleus. To characterize the subcellular localization of Igu/Dzip1, we expressed wild-type and mutant igu/dzip1 in cultured cells. Wild type Igu/Dzip1 is present in the cytoplasm and is found at especially high levels in large vesicles (Fig. 7A). These vesicles correspond to lysosomes and/or endosomes based on the co-localization with the lysosomal protein Lamp1 (Hughes and August, 1981) (Fig. 7B,C). By contrast, the mutant Igu/Dzip1 protein, which lacks the PEST sequences but retains the NLS, is strongly enriched in nuclei (Fig. 7D-F). Because a single zinc-finger domain does not bind to DNA, it is likely that Igu/Dzip1 is involved in protein-protein interactions. Indeed, human DZIP1 has been suggested to interact with DAZ, a protein required for spermatogenesis (Moore et al., 2003). Therefore, our results raise the possibility that Igu/Dzip1 might regulate the stability or the nuclear translocation of other proteins, including Gli proteins or other components of the Hh signaling pathway.

Igu is a permissive factor in Hh signaling

To further confirm that dzip1 is the gene mutated in igu, and to gain insights into the molecular nature of the igu mutations, we injected dzip1 mRNAs into wild-type and igu mutant embryos (Fig. 8). Injection of wild-type dzip1 mRNA (50 pg) into igu embryos completely recovered the normal expression of Hh target genes (A-E), including: ptc1 expression at 14 hpf (A), nk2.2 expression at 30 hpf (B), ptc1 expression at 30 hpf (C, D) and en1 at 30 hpf (E) (compare with Fig. 1). Wild-type embryos injected with wild type igu/dzip1 mRNA did not show any defects in Hh target gene expression (F-J). (K-T) Injection of mutant mRNA that encodes truncated igu/dzip1 proteins. Expression of mutant Igu/Dzip1 does not affect Hh target gene expression in igu mutants (K-O) or wild-type embryos (P-T). (A,D,F,L,K,N,P,S) Dorsal views and other panels are side views. Expression patterns in A-J are identical to those seen in wild-type embryos, while those in K-O are identical to igu mutant embryos (see Fig. 1 for comparison).

Table 2. dzip1 mRNA rescues igu phenotypes

<table>
<thead>
<tr>
<th>RNA injected</th>
<th>ptc1 (12-14 hpf)</th>
<th>ptc1 (30 hpf)</th>
<th>en1 (30 hpf)</th>
<th>nk2.2 (30 hpf)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal igu like</td>
<td>Normal igu like</td>
<td>Normal igu like</td>
<td>Normal igu like</td>
</tr>
<tr>
<td>dzip1 50 pg</td>
<td>19 0 (0%)</td>
<td>145 1 (1%)</td>
<td>101 0 (0%)</td>
<td>132 2 (2%)</td>
</tr>
<tr>
<td>dzip1 (igu296e) 50 pg</td>
<td>19 5 (21%)</td>
<td>53 19 (26%)</td>
<td>36 11 (23%)</td>
<td>58 21 (27%)</td>
</tr>
<tr>
<td>dzip1 (igu296e) 250 pg</td>
<td>19 5 (21%)</td>
<td>23 7 (23%)</td>
<td>16 7 (30%)</td>
<td>22 5 (19%)</td>
</tr>
</tbody>
</table>

Embryos were obtained from igu+/+ incrosses. Expected genotypes were 25% igu+/+, 50% igu+/ and 25% homozygous wild type. Percentages in parenthesis represent the ratios of embryos with igu-type gene expression to the total embryo tested for respective gene expressions. All of these embryos were confirmed to have igu/igu genotype.
(Fig. 8A-E; Table 2), consistent with dzip1 being the gene disrupted in igu mutants. Despite the dramatic rescue of the igu phenotype, no effect was observed upon injection of dzip1 mRNA into wild-type embryos (Fig. 8F-J), suggesting that Igu/Dzip1 is a permissive factor in Hh signaling. Importantly, the injection of mutant dzip1 mRNA into igu embryos [(Fig. 8K-O) or into wild-type embryos (Fig. 8P-T)] did not alter the Hh target gene expression (Table 2). Even a large amount of wild-type or mutant dzip1 mRNA (250 pg) did not produce a dominant effect on Hh target gene expression (Table 2). Thus, Igu/Dzip1, while being required for the proper regulation of Hh signaling, is not sufficient to activate or repress Hh target genes, and is therefore a permissive factor in the Hh signaling cascade.

**Discussion**

**Igu is necessary for the Hh-dependent activation of Gli proteins**

The zebrafish Hh pathway mutants dtr/gli1, yog/gli2, syu/shh and con all lead to a loss of Hh target gene expression in both the neural tube and in somites. However, igu is unique among midline mutants in that Hh target gene expression is reduced in the ventral neural tube and brain, whereas in somites the expression of Hh target genes is expanded. Cyclopamine treatments showed that the Hh-dependency of target gene activation is lost in igu mutants. This is probably not due to the activation of a different signaling pathway, as a decrease in Gli activator function (dtr/gli1:igu double mutants) and/or an increase in Gli repressor function (yog/gli2:igu double mutants) suppressed all Hh target gene expression in igu mutants. These results suggest that Gli proteins are responsible for the ectopic expression of Hh target genes in igu mutants, and that the Gli protein functions upstream of Gli1 and Gli2 in the Hh signaling cascade. As Gli protein activities are regulated in response to Hh signals, the Hh-independent activation of target genes in igu mutants suggests that Igu is not a transcriptional co-repressor or co-activator of Gli proteins.

Our examination of compound mutant phenotypes also revealed that the activating ability of Gli proteins is generally reduced in igu mutants, despite the apparent upregulation of en1 and ptc1 expression in somites. This reduced Gli activator function explains the loss of Hh signaling in the neural tube of igu mutants and suggests that Igu/Dzip1 function is required for the full activation of Gli proteins in response to Hh signals.

**Both positive and negative regulation of Hh signaling is impaired in igu mutants**

Previous studies have shown that Hh signaling is tightly regulated in the embryo, with different intracellular mechanisms positively and negatively regulating Hh signal transduction. Hh signaling is positively regulated through the functions of Fu, Cos2 and Su(Fu) and the nuclear trafficking of Gli/Ci proteins (see Fig. 9) (Monnier et al., 1998; Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000; Murone et al., 2000; Stegman, et al., 2000; Wang et al., 2000). Negative regulation is thought to occur via PKA-mediated processing of Gli/Ci proteins into repressor isoforms in the absence of Hh ligands (LePage et al., 1995; Ohlmeier and Kalderon, 1998; Pan and Rubin, 1995; Hammerschmidt et al., 1996; Jiang and Struhl, 1998).

Although our analysis of compound mutants suggested that the overall Gli activating function is reduced in igu mutants, it is probably not due to the direct decrease of full-length Gli proteins. If igu mutations simply lead to a reduction of full-length Gli activators, then we would predict that injection of dnPKA would still induce Hh responsive gene expression in igu mutants as it does in wild-type embryos. However, we found that dnPKA injections did not induce Hh responsive genes in igu mutants. This suggests that the positive regulation of Gli proteins, which may be either a modification of Gli proteins or nuclear trafficking, is disrupted in igu mutants.

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**Fig. 9. Model of possible Igu function in Hh signaling.** (A) In wild-type embryos, Igu function is required for the positive and negative regulation of Gli protein function in response to Hh signals. Components in green have activating functions, and those in red have repressor functions. (B) Without the normal Igu/Dzip1 protein function (light blue), Hh-dependent regulation is lost and Gli proteins cannot fully activate the expression of Hh target genes. In addition, the negative regulation of Hh signaling is also reduced. igu mutations could act directly on PKA-mediated negative regulation of Hh signals (broken arrows and lines in B) and/or could act indirectly, possibly by causing the constitutive nuclear import of Gli proteins (thin arrows). The resulting constitutive, but weak, activating function of Gli proteins is sufficient to induce en1 and ptc1 in broad regions in somites. This same low level of activator function is not sufficient to activate genes such as nk2.2 in the ventral neural tube the transcription of which requires a higher level of Hh signals.
We also showed that \textit{igu} mutations reduce the ability of PKA to repress Hh target gene expression. \textit{igu} mutants were resistant to forskolin, which blocks Hh signaling by activating PKA. This reduced sensitivity to forskolin treatment cannot be simply explained by defects in the positive regulation of Gli proteins. In \textit{Drosophila}, it has been shown that mutations in PKA lead to the accumulation of Ci and the upregulation of Hh signaling (Ohmeyer and Kalderon, 1998). Likewise, the upregulation of target genes in \textit{igu} mutants may partly be due to a defect in the negative regulation of Gli proteins. Taken together, our analyses suggest that both the positive and negative regulation of Hh signaling is impaired in \textit{igu} mutants (Fig. 9).

\textbf{Igu protein structure and function}

We showed that the \textit{igu} gene encodes a single zinc-finger protein, Dzip1. Although \textit{igudzip1} is conserved among vertebrate species such as human, mouse, rat, chicken and frog, we could not identify a homologous gene in \textit{Drosophila}. Considering the high degree of conservation in the Hh signaling pathway during evolution, a functionally equivalent protein may present in invertebrates, though the sequence might be highly diverged. Intriguingly, human postaxial polydactyly type A2 (PAPA2), a congenital defect that is caused by the ectopic activation of Hh signaling (Villavicencio et al., 2000), maps to a region containing human DZIP1 at 13q32. DZIP1 might be the gene disrupted in PAPA2.

Single zinc-finger domain proteins are not thought to bind DNA, instead they are implicated in mediating protein-protein interactions. The presence of PEST domains in the Dzip1 protein sequence suggests that the protein may be a target for rapid degradation and is consistent with our observation that Igu/Dzip1 accumulates in lysosomes in cultured cells. These facts suggest the possibility that Igu/Dzip1 is involved in mediating the rapid turnover of interacting proteins, perhaps components of the Hh signaling cascade. However, the Igu/Dzip1 protein may not simply be a cytoplasmic protein, as it has a nuclear localization signal (NLS) within the N-terminal conserved region. Indeed, the truncated Igu/Dzip1 proteins encoded by \textit{igu} are enriched in nuclei in cultured cells, suggesting that the NLS is also functional. This finding raises the possibility that wild type Igu/Dzip1 proteins shuttle between the cytoplasm and the nucleus, and they could thus affect the nuclear import of Hh pathway proteins. Although Gli proteins are possible candidates for Igu/Dzip1 interacting partners, our preliminary data suggest that Gli proteins do not physically interact with Gli proteins in vitro, and they do not alter the transcriptional activation of Gli proteins in a luciferase reporter assay (H.S. and A.K., unpublished). Igu proteins might interact with other components of the Hh signaling pathway such as Fu, Cos2 or Su(Fu). Intriguingly, Su(Fu) is involved in the nuclear trafficking of Gli proteins and also contains a PEST sequence (Pham et al., 1995; Pearse et al., 1999). Moreover, it was recently shown that reducing Su(Fu) function in zebrafish using morpholino oligonucleotides resulted in the upregulation of \textit{En} expression in somites (Wolf et al., 2003). From these data, we speculate that Igu/Dzip1 might be involved in the regulation of the Su(Fu), Fu and Cos2 cytoplasmic protein complex that is known to regulate Gli protein activities.

Our results also revealed that both wild-type and mutant versions of Igu/Dzip1 proteins do not have a dominant function when overexpressed in wild-type embryos. This, combined with our mutant analysis, indicates that Igu/Dzip1 function is a permissive factor required for the regulation of Hh signaling, and that mutant Igu/Dzip1 proteins do not function dominantly to interfere with the regulation of Hh signaling. These results support the idea that Igu/Dzip1 function is directly required for the negative regulation of Gli proteins. However, we cannot exclude the possibility that Igu mutant proteins have a new function that is masked in injected embryos by the presence of the wild-type protein. In fact, nuclear localization of mutant Igu/Dzip1 proteins in vitro could suggest a gained function for the mutant Igu/Dzip1 proteins. Although we attempted to generate a loss-of-function phenotype by blocking Igu/Dzip1 translation with antisense morpholino oligonucleotides, these morpholinos produced no phenotype in wild-type embryos, and did not alter Hh target gene expression in \textit{igu} mutants. Considering the weak and ubiquitous expression of the \textit{igu} gene and the permissive nature of Igu/Dzip1, it is possible that small amounts of Igu/Dzip1 proteins are sufficient for normal development, and that morpholinos are therefore unable to block expression sufficiently to produce a phenotype. Further biochemical analysis of Igu/Dzip1 will be needed to fully elucidate its function.

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Research article


