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

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Summary

Members of the Hedgehog (Hh) family of intercellular signaling molecules play crucial roles in animal development. Aberrant regulation of Hh signaling in humans causes developmental defects, and leads to various genetic disorders and cancers. We have characterized a novel regulator of Hh signaling through the analysis of the zebrafish midline mutant *iguana* (*igu*). Mutations in *igu* lead to reduced expression of Hh target genes in the ventral neural tube, similar to the phenotype seen in zebrafish mutants known to affect Hh signaling. Contradictory at first sight, *igu* mutations lead to expanded Hh target gene expression in somites. Genetic and pharmacological analyses revealed that the expression of Hh target genes in *igu* mutants requires Gli activator function but does not depend on Smoothened function. Our results show that the ability of Gli proteins to activate Hh target gene expression in response to Hh signals is generally reduced in *igu* mutants both in the neural tube and in somites. Although this reduced Hh signaling activity leads to a loss of Hh target gene expression in the neural tube, the same low levels of Hh signaling appear to be sufficient to activate Hh target genes throughout somites because of different threshold responses to Hh signals. We also show that Hh target gene expression in *igu* mutants is resistant to increased protein kinase A activity that normally represses Hh signaling. Together, our data indicate that *igu* mutations impair both the full activation of Gli proteins in response to Hh signals, and the negative regulation of Hh signaling in tissues more distant from the source of Hh. Positional cloning revealed that the *igu* locus encodes Dzip1, a novel intracellular protein that contains a single zinc-finger protein-protein interaction domain. Overexpression of Igu/Dzip1 proteins suggested that Igu/Dzip1 functions in a permissive way in the Hh signaling pathway. Taken together, our studies show that Igu/Dzip1 functions as a permissive factor that is required for the proper regulation of Hh target genes in response to Hh signals.

Key words: Neural tube patterning, Muscle cell specification, Hedgehog signaling, Cyclopamine, cAMP-dependent kinase A, Forskolin, Midline mutant, Adaxial cells, *dzip1*, PEST sequence, Zebrafish

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; Koebernick 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 Chiang, 2000; Wang et al., 2000; Persson et al., 2002). Despite suggestions that proteolytic processing may regulate Gli2 and Gli3 function, the in vivo cleavage of these proteins has not been directly demonstrated.

Thus, in both vertebrates and invertebrates, Hh signaling controls the expression of target genes by modulating the activity of the downstream Gli/Ci transcription factors. Studies in *Drosophila* have identified a large number of proteins that are involved in the regulation of this Gli/Ci activity (reviewed by McMahon, 2000; Ingham and McMahon, 2001; Nybakken and Perrimon, 2002). Within target cells, Ci forms a protein complex with Fused kinase (Fu) (Monnier et al., 1998; Stegman et al., 2000) and Costal2 (Cos2) (Robbins et al., 1997; Sisson et al., 1997) that is tethered to microtubules. Activated Smo signals to this cytoplasmic protein complex, resulting in the release of Ci and active transport into the nucleus where it can activate Hh target genes (Ohlmeyer and Kalderon, 1998; Ding et al., 1999; Kogerman et al., 1999; Methot and Basler, 2000; Murone et al., 2000; Wang et al., 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 response to Hh ligands, a negative pathway that includes cAMP-dependent protein kinase (PKA) also regulates Hh signaling (Li et al., 1995; Pan and Rubin, 1995; Lepage et al., 1995; Hammerschmidt et al., 1996). In the absence of Hh ligands, PKA directly phosphorylates Ci to promote its proteolytic cleavage, generating the repressor isoform (Chen et al., 1998; Chen et al., 1999; Price and Kalderon, 1999). Studies in vertebrates have shown that these intracellular components of the Hh signaling pathway have been largely conserved through evolution (Hammerschmidt et al., 1996; Pearse et al., 1999; Kogerman et al., 1999; Ding et al., 1999; Murone et al., 2000).

Genetic studies of Hh signaling in zebrafish complement the analyses in fly and other vertebrate species, and provide an approach to look into the regulation of Hh signaling in vertebrates. 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* (*tbx392*), *yot* (*ty119*), *dtr* (*ts269*), *con* (*tm15a*) and *igu* (*tms79a* 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, z6624 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* allele, 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 instruction and injected into embryos (1- to 4-cell stage) that were obtained from $igu^{+/-}$ 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 dechorinated with pronase at 50% epiboly and placed in 6 ml of embryo medium (Westerfield, 1993) containing 50 μM of cyclopamine or the desired concentrations of forskolin. Surviving embryos were fixed and processed for in situ hybridization.

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′-CAGG-TGTCGTTTTCAGGGTTA-3′) for igu^{ts294e}, z1660 for yot^{ty119}, and z14475 for dtr^{ts269}.

Transfection and antibody staining

PCR amplified wild-type and mutant (*ts294e* 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; Sbrogna 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,A2-F2). 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*>*yot/gli2*>*dtr/gli1*>*syu/shh*>*igu*. 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 neural tube and negatively regulated by Hh signaling (Ekker 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 (MPs) and a population of fast fibers around the MPs 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. 1A4,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 MPs 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*, *dtr/gli1*, *yot/gli2* and *con* mutants. More specifically, *ptc1* expression is severely reduced in *con* mutants both in the neural tube and in somites (Fig. 1F6,F7), whereas it is less severely reduced in the neural tube in *syu/shh*, *dtr/gli1* and *yot/gli2* mutants (Fig. 1C6-E6,C7-E7). Intriguingly, in *yot/gli2* mutant embryos, the medial-ventral regions of somites retain *ptc1* (Fig. 1E7). This region corresponds to developing sclerotomal tissues (Morin-Kensicki and Eisen, 1997), which is also induced by Hh signals (Fan and Tessier-Lavigne, 1994). In marked contrast to the other Hh pathway mutants, *igu* mutants show expanded *ptc1*

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.

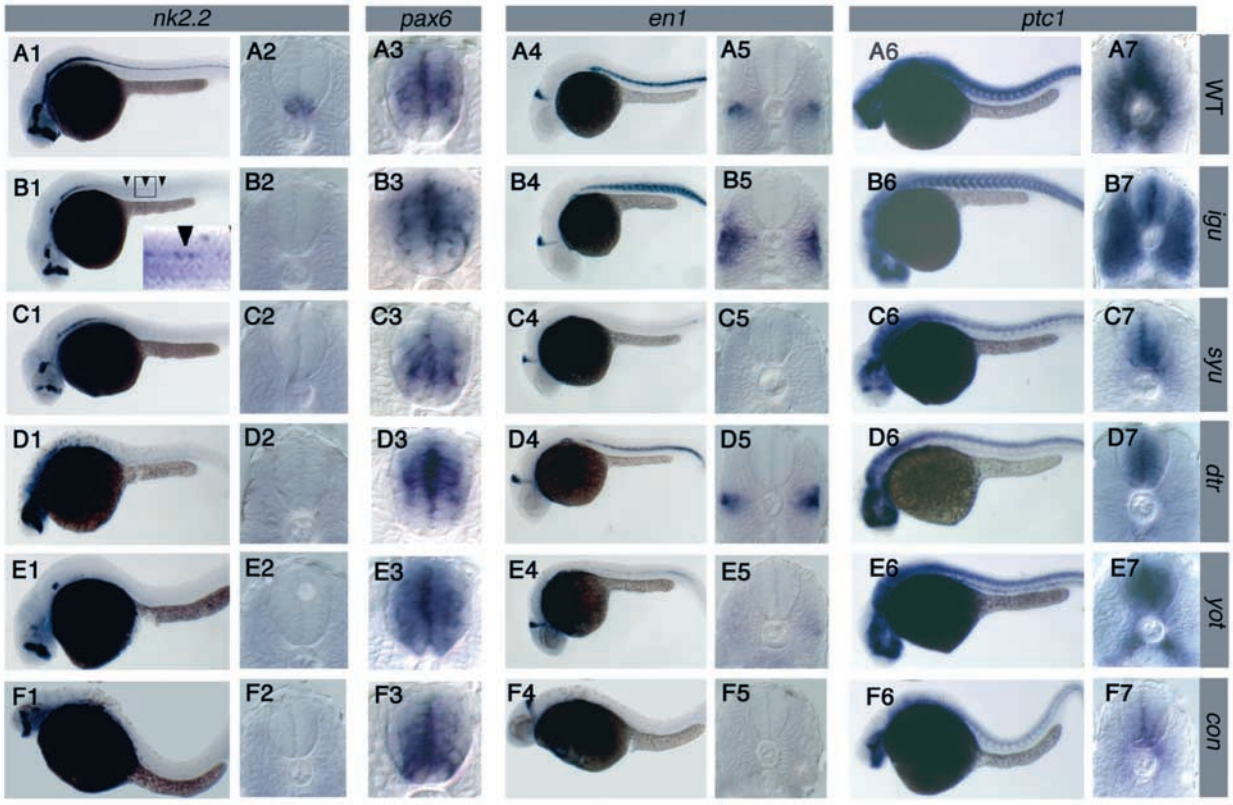

Fig. 1. Aberrant expression of Hh target genes in *igu* mutants and comparison with four other Hh signaling mutants. (A1-A7) Wild-type, (B1-B7) *igu*, (C1-C7) *syu/shh*, (D1-7) *dtr/gli1*, (E1-E7) *yot/gli2* and (F1-F7) *con* embryos at 30 hpf. (A1-F1,A2-F2) Expression of *nk2.2* in the ventral neural 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,A7-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/gli* 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.

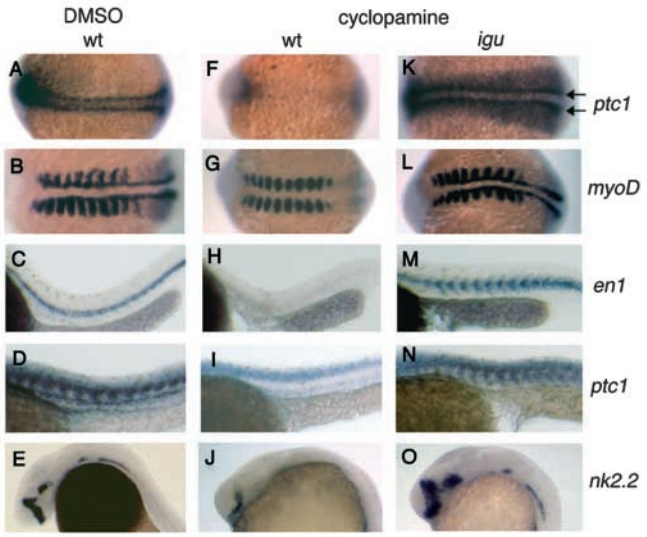

Fig. 2. Hh-independent target gene expression in *igu* embryos. (A-D) In control wild-type embryos treated with 5% DMSO, expression patterns of *ptc1* at 14 hpf (A), *myod* at 14 hpf (B), *en1* at 30hpf (C), *ptc1* at 30 hpf (D) and *nk2.2* at 30 hpf (E) are all normal. (F-J) Wild-type embryos treated with cyclopamine. Expression of *ptc1* at 14 hpf (F), adaxial *myod* at 14 hpf (G), *en1* at 30hpf (H), *ptc1* at 30 hpf (I) and *nk2.2* (J) at 30 hpf, is efficiently suppressed. Note that a low level of *ptc1* expression in the neural tube in I appears to be independent of Smo-mediated Hh signaling, since similar *ptc1* activation is seen in *smu/smo* mutants at 30 hpf (data not shown). (K-O) *igu* embryos treated with cyclopamine. A broad activation of *ptc1* expression in *igu* mutants at 14 hpf (K) is not affected by cyclopamine. Arrows indicate the adaxial cells. Expression of *myod* in adaxial cells is indistinguishable from normal wild-type embryos, and is not affected by cyclopamine (L). Likewise, *en1* (M) and *ptc1* (N) expression in 30 hpf embryos is not affected by cyclopamine. Despite the defect of *nk2.2* expression in *igu* mutants, the residual expression of *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.

Attenuated Gli activating function in *igu* mutants

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

To examine how more severe *gli* mutations affect the *igu* phenotype, we generated compound mutants with *yot/gli2* mutation. Existing *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 *yot/gli2* into *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 *yot/gli2*$^{-/-}$ homozygous mutants (Fig. 3M-O). The phenotype of *yot/gli2*$^{-/-}$;*igu*$^{-/-}$ homozygous double mutants is nearly identical to that seen in *yot/gli2*$^{-/+}$;*igu*$^{-/-}$ embryos (data not shown). These results suggest that Igu functions at the same level as, or upstream of, the Gli proteins in the Hh signaling cascade. More importantly, our results show that Hh target gene expression in *igu* mutants is sensitive to a decrease in Gli activator function. This suggests that the overall Gli mediated activating function of Hh target gene expression is reduced in *igu* mutants despite the ectopic activation of *en1* and *ptc1* genes in somites. We conclude that the normal Igu function is necessary for the full activation of Gli-mediated Hh signaling.

Table 1. Summary of epistasis analyses

	myod (12-14hpf)		*ptc1* (12-14hpf)		*nk2.2*		*ptc1*		
Injected mRNA	Up	*igu*	Up	*igu*	Up	*igu*	Up	*igu*	
shh (250 pg)	15	5 (25%)	16	4 (20%)	20	4 (17%)	20	10 (33%)	
dnPKA (250 pg)	26	9 (26%)	29	10 (26%)	60	21 (26%)	52	19 (27%)	
	myod (12-14hpf)		*ptc1* (12-14hpf)		*nk2.2*		*ptc1*		*en1*
Inhibitors	Down	*igu*	Down	*igu*	Down	*igu*	Down	*igu*	Down	*igu*
Cyclopamine (50 μM)	45	11 (20%)	33	10 (30%)	52	15 (22%)	62	22 (26%)	43	18 (30%)
Forskolin (300 μM)	45	0 (0%)		ND	64	0 (0%)	31	8 (20%)	66	1 (2%)
(150 μM)	28	1 (3%)		ND	45	8 (15%)	38	13 (25%)	44	5 (10%)
(75 μM)	18	6 (25%)		ND	19	8 (30%)	19	8 (30%)	22	7 (24%)

Embryos were obtained from *igu*/+ incrosses. Expected genotypes were 25% *igu*$^{-/-}$, 50% *igu*$^{-/+}$ and 25% homozygous wild type.
Up, respective Hh target genes are ectopically expressed. These embryos have +/+ or +/*igu* genotypes.
Down, Gene expression is downregulated.
igu, Hh target genes are expressed in the same pattern in *igu* mutants.
Percentages in parenthesis represent the ratios of embryos with *igu*-type gene expression to the total embryos tested for respective gene expressions. All of these embryos have *igu/igu* genotype. Note that *igu* embryos have a seemingly normal *myod* expression pattern.

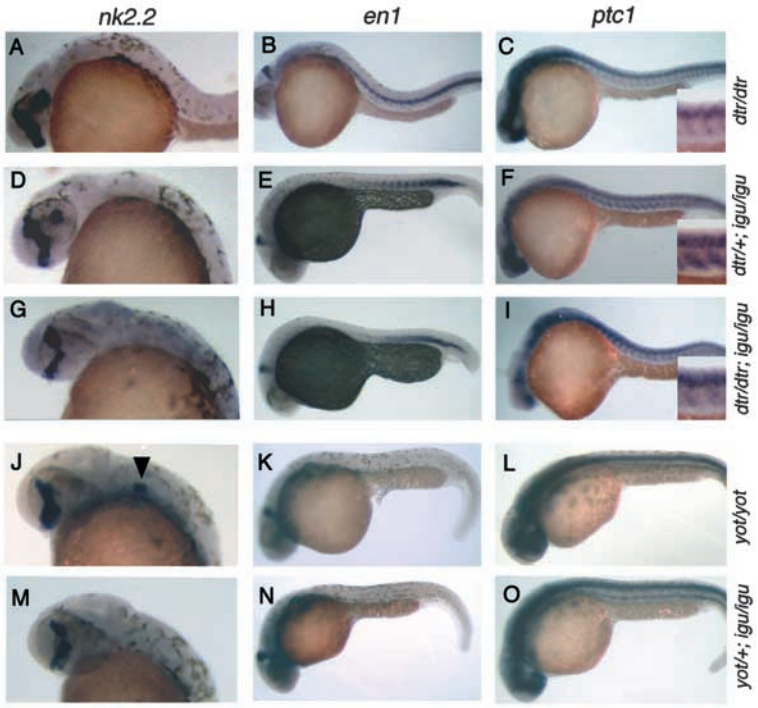

Fig. 3. Decreased Gli activator function and/or increased Gli repressor function represses ectopic Hh target gene expression in *igu* embryos. (A-I) Introduction of *dtr/gli1* mutant alleles into the *igu* genetic background suppressed ectopic Hh target gene expressions in somites. In *dtr/gli1*$^{-/-}$ embryos (A-C), the expression of *nk2.2* and *ptc1* is reduced, but *en1* expression is normal. In *dtr/gli1*$^{-/+}$;*igu*$^{-/-}$ embryos (D-F), *nk2.2* expression is similar to *dtr/gli1*$^{-/-}$ 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*$^{-/-}$ mutants; however, *en1* expression is reduced in anterior somites. In *dtr/gli1*$^{-/-}$;*igu*$^{-/-}$ 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*$^{-/-}$ 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*$^{-/-}$ embryos (J-L). An arrowhead marks a spot of *nk2.2* expression, which is consistently seen only in *yot/gli2*$^{-/-}$ embryos (J) but not in *yot/gli2*$^{-/+}$;*igu*$^{-/-}$ 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*

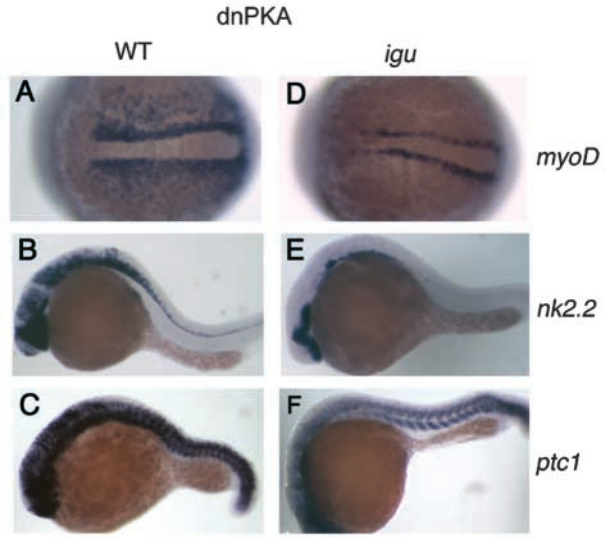

Fig. 4. Inhibition of PKA does not activate Hh target genes. (A-C) Injection of 250 pg of dnPKA mRNA into wild-type embryos induced the ectopic expression of *myod* at 12 hpf (A), *ptc1* at 30 hpf (B) and *nk2.2*at 30 hpf (C). (D-F) In *igu* embryos, overexpression of dnPKA did not induce the expression of *myod* (D), *nk2.2* (E) and *ptc1* (F), and Hh target gene expression is similar to that in *igu* mutants. Genotypes were confirmed by PCR after in situ analysis.

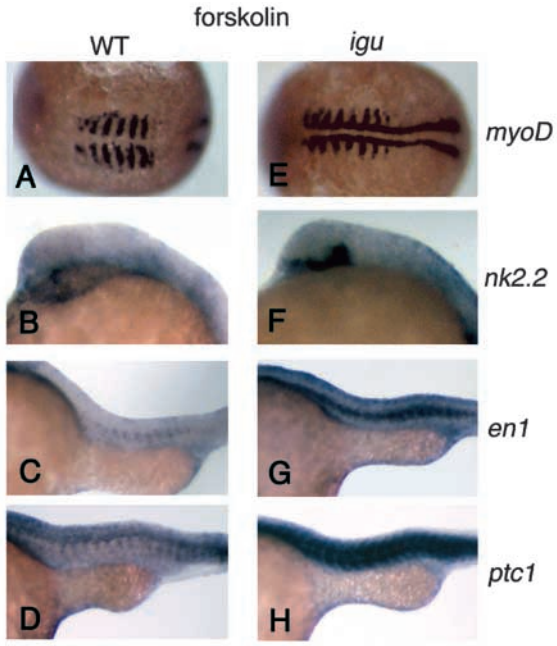

Fig. 5. Activation of PKA does not efficiently suppress Hh target gene expression. (A-D) In wild-type embryos, the expression of *myod* at 14 hpf (A), *nk2.2* at 30 hpf (B), *en1* at 30 hpf (C) and *ptc1* at 30 hpf (D) is repressed by 75 μM forskolin treatment. (E-H) In *igu* embryos, treatment with 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

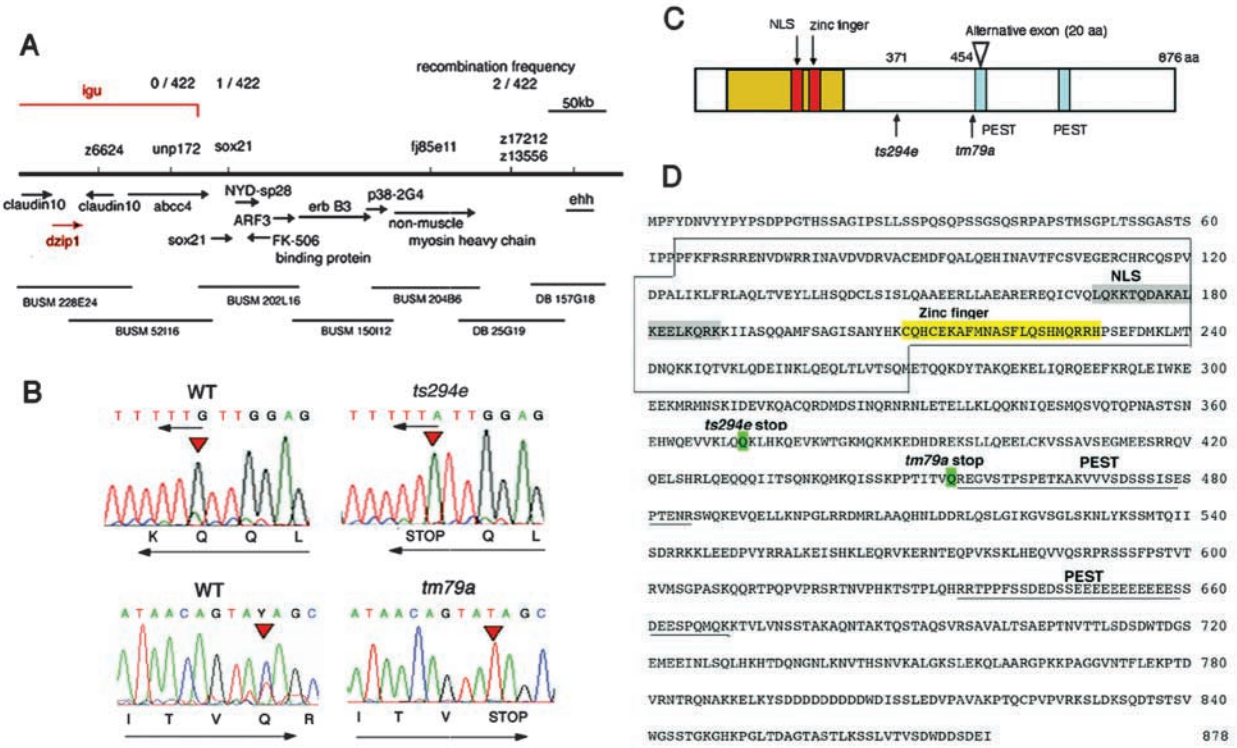

Fig. 6. The zinc-finger gene *dzip1* is disrupted in *igu* mutants. (A) Genetic map of *igu* mutation relative to known genes in the region. P1 artificial chromosome (PAC) clones (below) and predicted ORFs (arrows in the middle) are shown. (B) Sequence ferograms showing nonsense mutations in *igu*^{ts294e} and *igu*^{tm79a} alleles. (C) Schematic view of the structure of predicted Igu/Dzip1 protein. A conserved domain (yellow box) has 51% identity with the human DZIP1. Putative nuclear localization signal (NLS), zinc finger (red boxes) and PEST domains (blue boxes) are shown. (D) Amino acid sequence of zebrafish Igu/Dzip1 protein. The NLS (gray), zinc finger (yellow) and positions of nonsense mutations (green) are highlighted. The N-terminal conserved domain is boxed. PEST sequences are underlined.

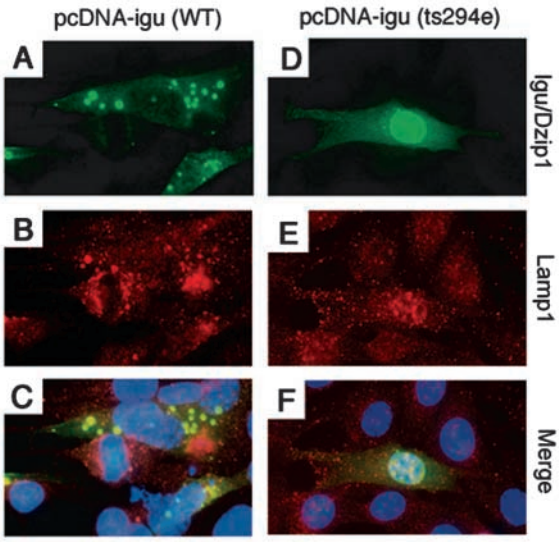

Fig. 7. Subcellular localization of Igu/Dzip1 proteins in cultured cells. (A-C) Transfection of a construct encoding the His-tagged Igu/Dzip1 protein into NIH3T3 cells. Wild-type Igu/Dzip1 proteins (A) are located in the cytoplasm and enriched in punctate vesicles. These vesicles are also enriched with the lysosomal protein Lamp1 (B, C). (D-F) Transfection of a construct encoding the His-tagged Igu/Dzip1(*ts294e*) mutant protein. The truncated mutant Igu/Dzip1 proteins (D) are distributed in the cytoplasm and enriched in nuclei. (C,F) Merged images, in which nuclei are stained with 4′,6-diamidino-2-phenylindole (DAPI).

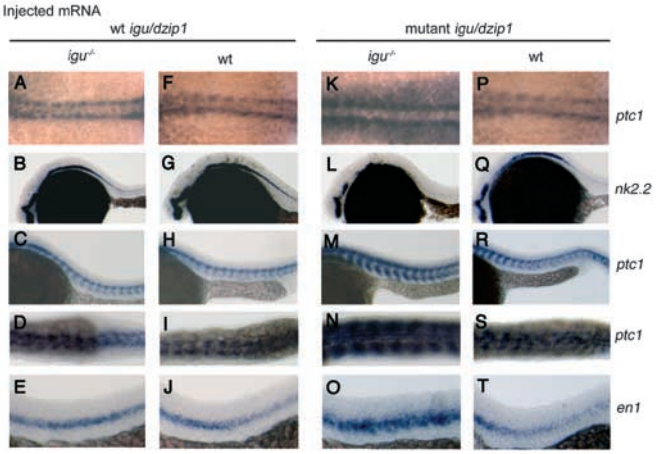

Fig. 8. Rescue of *igu* mutant phenotype by *igu/dzip1* mRNA and permissive nature of Igu/Dzip1 proteins. (A-J) Injection of wild-type *igu/dzip1* mRNA. Nearly all *igu* embryos injected with wild-type *igu/dzip1* mRNA completely recovered the normal expression of Hh target genes (A-E), including: *ptc1* 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,I,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).

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 dramatically rescued the *igu* phenotypes both in the neural tube and somites of almost all mutant embryos

Table 2. *dzip1* mRNA rescues *igu* phenotypes

	ptc1 (12-14 hpf)		*ptc1* (30 hpf)		*en1* (30 hpf)		*nk2.2* (30 hpf)	
RNA injected	Normal	*igu* like	Normal	*igu* like	Normal	*igu* like	Normal	*igu* like
dzip1 50 pg	19	0 (0%)	145	1 (1%)	101	0 (0%)	132	2 (2%)
dzip1 (*igu*^{ts294e}) 50 pg	19	5 (21%)	53	19 (26%)	36	11 (23%)	58	21 (27%)
dzip1 (*igu*^{ts294e}) 250 pg	19	5 (21%)	23	7 (23%)	16	7 (30%)	22	5 (19%)

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*, *yot/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 (*yot/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 Igu 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; Ohlmeyer and Kalderon, 1998; Pan and Rubin, 1995; Hammerschmidt et al., 1996; Jiang and Struhl, 1998).

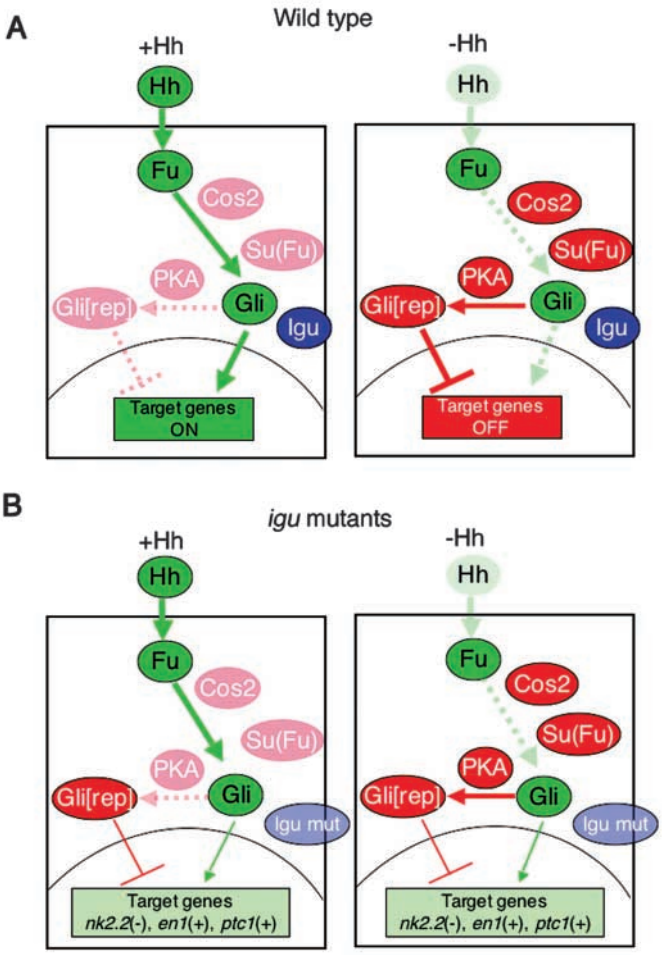

Fig. 9. Model of possible Igu function in Hh signaling. (A) In wild-type embryos, Igu function is required for the positive and negative regulation of Gli protein function in response to Hh signals. Components in green have activating functions, and those in red have repressor functions. (B) Without 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.

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.

We also showed that *igu* mutations reduce the ability of PKA to repress Hh target gene expression. *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 *Drosophila*, it has been shown that mutations in PKA lead to the accumulation of Ci and the upregulation of Hh signaling (Ohlmeyer and Kalderon, 1998). Likewise, the upregulation of target genes in *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 *igu* mutants (Fig. 9).

Igu protein structure and function

We showed that the *igu* gene encodes a single zinc-finger protein, Dzip1. Although *igu/dzip1* is conserved among vertebrate species such as human, mouse, rat, chicken and frog, we could not identify a homologous gene in *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 *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 Igu 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 En expression in somites (Wolff 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 *igu* mutants. Considering the weak and ubiquitous expression of the *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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