Iguana encodes a novel zinc-finger protein with coiled-coil domains essential for Hedgehog signal transduction in the zebrafish embryo

Christian Wolff,1,2,4 Sudipto Roy,1,3,4 Katharine E. Lewis,1,5 Heike Schauerte,2 Gerd Joerg-Rauch,2 Annette Kirn,2 Christian Weiler,2 Robert Geisler,2 Pascal Haffter,2 and Philip W. Ingham1

1Center for Developmental Genetics, School of Medicine and Biomedical Sciences, University of Sheffield, Western Bank, Sheffield S10 2TN, UK; 2Max-Planck-Institut für Entwicklungsbiologie, Abteilung III, Tübingen 72076, Germany; 3Institute of Molecular and Cell Biology, Singapore 117609, Republic of Singapore

Signaling by lipid-modified secreted glycoproteins of the Hedgehog family play fundamental roles during pattern formation in animal development and in humans; dysfunction of Hedgehog pathway components is frequently associated with a variety of congenital abnormalities and cancer. Transcriptional regulation of Hedgehog target genes is mediated by members of the Gli zinc-finger transcription factors. The relative nuclear concentrations of Gli activator (Gliact) and repressor (Glirep) forms, together with their nucleocytoplasmic trafficking, appear to be critical determinants for target gene expression. Whereas such stringent controls of Gli activity are critical in ensuring appropriate levels of pathway activation, the mechanisms by which these processes are regulated remain inadequately understood. Here, using genetic analysis, we show that the zebrafish iguana gene product acts downstream of the Smoothened protein to modulate Gli activity in the somites of the developing embryo. Positional cloning reveals that iguana encodes the zebrafish ortholog of Dzip1, a novel zinc-finger/coiled-coil domain protein that we show can shuttle between the cytoplasm and nucleus in a manner correlated with Hedgehog pathway activity.

Keywords: Iguana; Gli; hedgehog signaling; zebrafish; muscle; Dzip1

Supplemental material is available at http://www.genesdev.org.

Received December 23, 2003; revised version accepted April 29, 2004.

During animal development, cell fates are specified in response to inductive signals that emanate from discrete sources within the developing embryo. A rather limited number of families of signaling proteins have been implicated in these inductive processes, the complexity of the range of responses they induce reflecting the importance of both cellular context and signal strength. The multiple roles of Hedgehog (Hh) family proteins in animal development exemplify the way in which the same signals can be reiteratively deployed to direct a variety of developmental processes (for review, see McMahon et al. 2003). The myotome of the zebrafish embryo presents a relatively simple paradigm for the analysis of cell-fate specification by Hh signaling, having the advantage of being readily amenable to genetic analysis. Using mutants and morphants (morpholino antisense oligonucleotide-injected embryos) to manipulate Hh-signaling activity, we have previously shown that different levels of Hh signaling specify distinct muscle-cell identities within the somites of the zebrafish embryo in a defined temporal sequence (Wolff et al. 2003). Early in embryogenesis, so-called adaxial cells that flank the notochord segregate into two different kinds of slow-twitch muscle fibers that differentiate in distinct regions of the myotome. The superficially located slow-twitch muscle fibers (SSFs) develop in response to low levels of Hh signaling emanating from the notochord, whereas medially located MP cells, characterized by their expression of high levels of the homeobox containing Engrailed (Eng) proteins, are specified by maximal levels of Hh activity. Later in development, myoblasts of the fast-twitch lineage located close to the notochord and surrounding the MPs are induced to express low levels of Eng proteins and differentiate into MFFs (medial fast fibers) in response to intermediate levels of Hh signaling (Wolff et al. 2003).
have been shown to inactivate the zebrafish (Brand et al. 1996; van Eedeen et al. 1996) that was confirmed by studies of the midline class of mutants in the 2003; Motoyama et al. 2003). This picture has been converted into the presence of three homologs of cip1, a serpentine protein essential for transduction of all Hh-signaling activity. Activated Smo signals to the transcription factor Cubitus interruptus [Ci] via a direct interaction with Costal-2, a component of a multimeric complex that includes Ci and the serine threonine kinase Fused [Fu], for review, see Kalderon 2004]. Ci acts as a bipotential transcription factor that can repress as well as activate Hh target genes. The repressor form is generated by limited proteolysis of the full-length protein, a process promoted by PKA-mediated phosphorylation, that is, in turn, potentiated by interaction with the Cos/Fu complex. Interaction with Costal-2 and a fourth intracellular component, Suppressor of fused [Su(fu)] also regulates the nuclear import of both the activator and repressor forms of Ci. It is the balance between these activator and repressor forms of Ci within the nucleus of a given cell that ultimately determines the specific target genes that the cell expresses in response to a particular level of Hh-signaling activity.

Targeted inactivation of murine orthologs of the Drosophila Hh-signaling pathway components has established that the mechanism of Hh signaling has been highly conserved through evolution (Chiang et al. 1996; Goodrich et al. 1997; Zhang et al. 2001; Caspary et al. 2002; Kawakami et al. 2002; Ma et al. 2002). Of the principal components of the pathway that identified in the fly, only vertebrate orthologs of costal-2 have yet to be described. One other notable difference, however, is the presence of three homologs of ci in vertebrates, designated Glil1, Glil2, and Glil3, each of which seems to perform a subset of the functions subsumed by ci [Mo et al. 1997; Matise et al. 1998; Park et al. 2000; Buttitta et al. 2003; Motoyama et al. 2003]. This picture has been confirmed by studies of the midline class of mutants in the zebrafish [Brand et al. 1996; van Eedeen et al. 1996] that have been shown to inactivate the shh, [Schuarte et al. 1998], disp1 [Nakano et al. 2004], smo [Chen et al. 2001; Varga et al. 2001], glil1 [Karlstrom et al. 2003], and glil2 [Karlstrom et al. 1999] genes. In addition, the use of morpholinos has provided the first in vivo evidence for a conserved function of Fu and Su(fu) in regulating Gli activity [Wolff et al. 2003].

Previous studies of another zebrafish midline mutant, iguana (igu), have highlighted several phenotypic traits that it shares with the glil1 [dtr] and glil2 [yot] mutants [Karlstrom et al. 1996; Kondoh et al. 2000; Odenthal 2000], suggesting a link between igu function and Gli activity. Here, using genetic analysis, we adduce evidence that igu acts downstream of Smo to regulate Gli protein function. By adopting a positional cloning approach, we have isolated the igu gene and found that it encodes a novel protein with a single zinc-finger and coiled-coiled domain that can shuttle between the cytoplasm and the nucleus. We discuss the role of igu in Hh signaling in the light of these findings.

Results

Igu mutant embryos display aberrant Hh pathway activity

In most developmental and pathological contexts, the response of cells to Hh signaling is marked by an up-regulation of ptc transcription. In the zebrafish embryo, ptc1 up-regulation is prominent in the adaxial slow muscle precursors of the paraxial mesoderm as well as in the ventral portion of the neural tube [Concordet et al. 1996], two cell populations in which Hh signaling plays a major role in cell-fate specification. We found that embryos mutant for igu display a significant expansion of the spatial domain of ptc1 expression in the paraxial mesoderm from the end of gastrulation onward [Fig. 1A,B]. Despite this ectopic expression, however, the levels of ptc1 transcript accumulation, both in the adaxial cells and the neurectoderm of igu mutants, are initially reduced relative to their wild-type siblings [Fig. 1A,B]. During somitogenesis, however, the ectopic expression becomes progressively more pronounced, such that by 24-h post fertilization [hpf], ptc1 transcript levels are substantially elevated and the expression domain is expanded both in the somites and in the neural tube [Fig. 1C,D; data not shown]. Reflecting this altered pattern of ptc1 expression, the specification of Hh-dependent muscle-cell types is also aberrant in igu mutants. SSFs, normally induced by relatively low levels of Hh activity are reduced in numbers, whereas MPs, induced by high levels of Hh activity, are completely absent from the anterior-most somites [Fig. 1E,F; data not shown]. In the remainder of the somites, MPs are reduced in number and exhibit lowered levels and delayed onset of Eng expression [Fig. 1G,H]. In contrast to this depletion of Hh-dependent slow lineage cell types, MFFs, the later Hh-induced subset of fast-twitch fibers, are substantially increased in numbers [Fig. 1G–J]. Hence, mutation of the igu gene has two diametrically opposed effects on the specification of cell types induced by different levels of Hh at distinct developmental stages—a reduction in those induced early, with a partial elimination of those requiring maximal signaling activity, followed by a uniform expansion of later induced cell-types requiring intermediate levels of Hh.

Igu mutations act downstream of sonic hedgehog, smoothened and Protein Kinase A

The loss of Hh-dependent cell types could reflect a reduction of signaling activity in igu embryos or an inability of mutant cells to respond to Hh signals. To distinguish between these possibilities, we analyzed the response of igu mutants to ectopic Shh activity. In wild-
type embryos injected with Shh mRNA, most of the cells of the myotome are transformed to the MP fate (Currie and Ingham 1996; Hammerschmidt et al. 1996; Bladgen et al. 1997; Du et al. 1997); in contrast, the myotomes of similarly injected igu embryos show no such transformation [Fig. 2A–C]. Thus, the ability of cells to respond to Shh activity appears to be compromised in igu mutants.

Despite the partial loss of some Hh-dependent muscle cell types in igu mutants, the very same cell types that are completely absent from embryos lacking Shh activity [MPs and MFFs] or Smo activity [all slow-lineage muscles and MFFs], can be partially restored in syu or smu embryos that are also mutant for igu. Moreover, the late gain of Hh-signaling phenotype exemplified by the expansion in numbers of MFFs is unaffected by the loss of Shh or Smo activity from igu embryos [Fig. 2G–L; data not shown]. Taken together, these data imply that Igulo acts downstream of Igulo and Igulo to modulate the activity of the intracellular components of the signaling pathway in responding cells. Consistent with this interpretation, we have found that the igu mutation behaves cell autonomously in genetically mosaic embryos [data not shown].

The activity of the Gli transcription factors is negatively regulated by PKA, which acts to promote the cytoplasmic sequestration of Gli1 and generate the repressor forms of Gli3 and most likely, Gli2. Accordingly, expression of a constitutively active form of PKA (cPKA) in the zebrafish embryo blocks all responses to Hh signaling, whereas inhibition of PKA leads to ectopic activation of Hh target genes and the differentiation of Hh-dependent cell types (Concordet et al. 1996; Hammerschmidt et al. 1996; Fig. 3A). Expression of cPKA in igu mutant embryos suppresses the differentiation of all MFFs as well as MPs [Fig. 3B], implying that the gain of Hh-signaling phenotype is dependent upon Gli activity. In contrast, igu embryos are insensitive to the effects of inhibiting PKA as revealed by their failure to up-regulate ptc1 expression in early somitogenesis stage embryos [Fig. 3, cf. C and D] and by their lack of supernumary MPs [data not shown]. Thus, mutation of igu is refractory to the hyperactivation of Gli proteins induced by blockade of PKA activity, suggesting that igu regulates Gli activity downstream of PKA.

igu mutants have distinct affects on Gli1 and Gli2 activities

To elucidate the effects of igu on Gli activity further, we manipulated the expression of Gli1 or Gli2 indepen-
dently in \textit{igu} mutant embryos. In the presence of wild-type \textit{igu} activity, loss of either Gli1 or Gli2 alone has little or no impact on the differentiation of the myotome; however, simultaneous elimination of both Gli1 and Gli2 results in complete loss of all Hh-dependent muscle fiber-types, revealing a critical requirement for one or other activating forms of the two proteins in this tissue [Wolff et al. 2003]. Strikingly, embryos double mutant for \textit{igu} and the Gli1 loss-of-function mutant \textit{dtr}, [Karlstrom et al. 2003], lack MFFs and almost all slow-twitch muscle cells [Fig. 4A–D]. Moreover, specification of MPs is inhibited even in \textit{igu} embryos that are hetero-

Figure 2. \textit{igu} mutants are refractory to modulations in Hh signaling activity. (A) A Shh overexpressing wild-type embryo showing expanded MP cell population labeled with anti-Eng (green, $n = 51/57$). (B) A Shh overexpressing \textit{igu} embryo labeled with anti-Eng showing no alteration in cell fates in the myotome ($n = 23/23$). (C) The embryo depicted in B, showing the nuclear localized β-galactosidase (red) tracer, whose mRNA was coinjected with that of Shh to ensure proper distribution of injected mRNAs. (D) Pattern of Eng expression in the myotome of a wild-type embryo revealed by histochemistry with anti-Eng antibodies. MPs [long arrows] and MFFs [short arrows] are indicated. (E) Eng expression in the myotome of an \textit{igu} mutant embryo. Note the expansion of MFFs [short arrows] and a reduction of MPs [long arrows]. (F) Similar pattern of Eng expression in the myotome of an \textit{igu} mutant embryo. (G) Complete absence of Eng expression from the myotome of a \textit{syu} mutant embryo. (H) \textit{igu}-like pattern of Eng expression in the myotome of a \textit{syu} gil29\textsuperscript{<del>eo\</del>} double-mutant embryo. MPs [long arrow] and MFFs [short arrow] are indicated. (I) Complete absence of Eng expression from the myotome of a \textit{smu} \textit{igu} mutant embryo. (J) \textit{igu}-like pattern of Eng expression in the myotome of a \textit{smu} \textit{igu} double-mutant embryo. MPs [long arrow] and MFFs [short arrow] are indicated. (K) Loss of all Eng expression from the myotome of a wild-type embryo treated with cyclopamine ($n = 12/12$). (L) Eng expression is unaffected in an \textit{igu} mutant embryo when treated with cyclopamine ($n = 7$). All panels show lateral views of 24 hpf stage embryos.

Figure 3. The \textit{igu} mutation is epistatic to PKA. (A) A wild-type embryo injected with mRNA encoding cPKA and labeled with anti-Eng antibodies showing loss of Eng expression from the myotome ($n = 38/47$). (B) Similar effect of cPKA overexpression in an \textit{igu} mutant embryo ($n = 17/21$). (C) Ectopic expression of \textit{ptc1} in a wild-type embryo in response to dnPKA overexpression ($n = 34/34$, cf. Fig. 1A). (D) The pattern of \textit{ptc1} expression in an \textit{igu} embryo remains unaltered on dnPKA overexpression ($n = 16/16$, cf. Fig. 1B). (A, B) Lateral views of 24 hpf embryos. (C, D) Dorsal views of 1–2 somite-stage embryos.
zygous for dtr (igu, dtr/+ embryos, Fig. 4E,F). It follows that Gli2 function is unable to compensate for the reduction of Gli1 or loss of Gli1 activity in igu mutants. Abrogation of Gli2 activity [by MO-mediated translational inhibition] in igu mutant embryos, in contrast, has relatively little effect on the response of myotomal cells to Hh signaling. SSFs still differentiate, albeit in slightly reduced numbers, whereas the differentiation of MP cells is affected only in so far as they lose their medial location within the somite and appear contiguous with the SSFs [Fig. 4L]. This implies that Gli1 function not only compensates for loss of Gli2 activity in igu mutants, but is also responsible for the ectopic MFFs characteristic of the mutant embryos. Notably, the level of Eng expression in the MFFs of igu;gli2MO embryos is increased relative to that normally apparent in igu mutant embryos [cf. Figs. 4L and 1J]. This may reflect the reduction of Gli2_rep activity due to the MO-mediated translational inhibition.

The truncated forms of the Gli2 protein encoded by the yot mutant alleles resemble the endogenous Gli1_rep form and behave as dominant repressors of Hh target gene expression; consequently, yot/+ heterozygotes are associated with a mild, but discernible reduction of Hh-signaling activity [van Eeden et al. 1996; Karlstrom et al. 1999] and exhibit sporadic loss of MP cells from the myotome [Fig. 4C; see also Wolff et al. 2003]. Remarkably, this antimorphic effect of yot is significantly enhanced by the igu mutation; yot/+;igu embryos completely lack all Hh-dependent muscle cells and are indistinguishable from igu;yot double-mutant homozygotes [Fig. 4H].

igu mutants are hypersensitive to variations in the levels of Su[fu]

Gli protein function is modulated by the Su[fu] protein, which acts both to sequester Glis in the cytoplasm and to inhibit their transcriptional activation activity in the nucleus [for review, see Ingham and McMahon 2001]. Consistent with these roles, we have previously shown that morpholino-mediated inhibition of Su[fu] expression in the zebrafish embryo causes a significant increase in MFFs, an effect that can be attributed to an increase in
Gli1 activity and a loss of Gli2 rep activity (Wolff et al. 2003). Injection of Su(fu) MOs into igu mutants causes a further expansion in the numbers of MFFs, such that they now occupy most of the myotome (Fig. 5, cf. A and B). Conversely, overexpression of Su(fu), which in wild-type embryos has no discernable effect on muscle differentiation (Fig. 5C,D), results in the near elimination of all Hh-dependent muscle cell types from igu mutant embryos [Fig. 5E,F].

Igu encodes a novel protein with a zinc-finger and coiled-coil domain

To elucidate the molecular basis of the igu mutant phenotype, we adopted a positional cloning strategy to isolate the gene. Recombination analysis localized igu to linkage group 6 (LG6), in the vicinity of SSLP markers z17212, z6624, and z9870 (Fig. 6A). Using these markers as a starting point for a genomic walk, we isolated overlapping PAC and BAC clones and constructed a contig comprising several hundreds of kilobases (Fig. 6A). Fine mapping utilizing additional markers derived from the genomic clones placed igu within a critical interval on PAC clone A5 and an overlapping BAC clone D13 (Fig. 6A). Injection of BAC D13 (but not of PACs A1, A5, or BAC C16) into newly fertilized eggs resulted in a partial rescue of the lateral floorplate (LFP; Odenthal et al. 2000) defect manifest in igu mutants [Fig. 6B–D], indicating that the gene mutated in igu is located on this particular genomic fragment. Annotation of this BAC sequence predicted a single ORF in the candidate interval, encoding a protein with a C2H2-type zinc finger at its N terminus [residues 209–230], a putative bipartite nuclear localization signal [NLS, residues 172–188], a leucinerich motif matching the nuclear export signal [NES] consensus sequence LXX_{1-3}LXX_{2-3}LXX [residues 532–541], and a significant number of coiled-coil motifs distributed throughout the remainder [Fig. 6H, Supplementary Fig. 1]. Genome sequence database searches revealed extensive synteny between the genomic region of LG 6 containing igu and the region of human chromosome 13 containing the gene encoding DAZ-interacting protein 1 (DZIP1; Moore et al. 2003), a novel protein of unknown biological function exhibiting significant sequence identity to Igu [Supplementary Fig. 1; data not shown]. We isolated a full-length cDNA clone encoding the zebrafish Dzip1 homolog; injection of synthetic mRNA derived from this clone [fused at the 5’ end with the cDNA encoding GFP, Fig. 6I] fully rescued the muscle cell-type specification defects of igu mutants [Fig. 6E]. Sequence analysis of the two original mutant alleles of igu, together with a third newly recovered in our laboratory, revealed the presence of premature stop codons in all three mutations predicted to result in C-terminal truncations of the Dzip1 protein [Fig. 6H; Supplementary Figs. 1, 2], confirming that igu encodes zebrafish Dzip1. Injection of mRNA derived from a mutant cDNA encoding a truncated form of Igu [corresponding to the igu(294G) allele] fused to GFP [Fig. 6J] does not rescue the igu mutant phenotype [Fig. 6G].

Igu alleles represent amorphic mutations that completely abolish the function of the protein

All three igu mutant alleles analyzed are predicted to encode truncated proteins retaining the zinc finger and NLS, raising the possibility that their aberrant nuclear localization could contribute to the igu mutant phenotype. To investigate this possibility, we sought to establish the phenotypic consequences of complete loss of igu expression using MO-mediated “knock-down”. MOs targeted against the igu translation initiation site surprisingly had no discernible effect on injected embryos [data not shown]. We therefore tested two different splice site-targeted antisense MOs (see Materials and Methods) predicted to induce exclusion of exon2 or exon3 [encoding
regions N-terminal to the zinc-finger domain and the zinc-finger domain, respectively) during processing of the nascent igu mRNA (Fig. 7A). Injection of either one or a combination of these MOs into wild-type embryos phenocopied all aspects of the Hh-signaling defects apparent in igu mutants, and like the igu alleles, also effectively suppressed the loss of Smo activity induced by cyclopamine [Fig. 7B,C; data not shown]. In addition, injection of the MOs into the iguts294e embryos themselves had no effect on their mutant phenotype (data not shown), indicating that this does not depend upon the expression of the truncated form of the protein encoded by the mutant allele. Consistent with this, we found that injection of mRNA encoding the GFP-tagged truncated form of the protein into wild-type embryos had no effect on their pattern of muscle differentiation [Fig. 6F].

Figure 6. Positional cloning of the igu locus. [A] A genetic map of the igu locus on LG 6, showing the positions of the SSLP (z), SSCP, and SNP markers, genomic clones, and the order of predicted ORFs spanning the interval. Numbers of recombinants and recombination frequencies [in centimorgans [cM]] with markers from the left (north, green) and the right (south, red) directions are displayed. [B] LFP cells of a wild-type embryo showing expression of fkd4. Note that fkd4 is expressed in the LFP as well as in the medial floor plate [MFP] that is flanked by the LFP. [C] In an igu mutant embryo, LFP cells are absent and fkd4 expression remains restricted to a single row of MFP cells. [D] Restoration of patches of fkd4 expressing LFP cells [arrows] in an igu mutant embryo on injection of BAC D13 (n = 16/25). [E] Wild-type like pattern of Eng expressing muscle cells in the myotome of an igu embryo injected with synthetic mRNA derived from a full-length wild-type igu cDNA fused at the 5’ end to that encoding GFP (n = 19/23, cf. Fig. 2D,E). [F] A wild-type embryo injected with mRNA encoding the mutant Igu protein corresponding to the iguts294e allele tagged N-terminally with GFP showing no changes in the pattern of Eng expression (n = 50). [G] Pattern of Eng expression remains unaffected in iguts294e embryos injected with the same mutant mRNA (n = 14). [H] Diagrammatic representation of the wild-type Igu protein. The areas shaded in dark and light gray represent >50% and >25% conservation, respectively, with the human DZIP1 protein [see also Supplementary Fig. 1]. The N-terminal zinc finger is depicted in light blue and the C-terminal coiled-coil domains in dark blue. The approximate positions where the protein truncates in the three different igu alleles, as well as the position of an alternatively spliced exon [black box] is indicated [see also Supplementary Fig. 1]. For reasons of clarity, the putative NES, NLS, and coiled-coil domains around the zinc finger at the N terminus are not depicted. [I] Diagrammatic representation of wild-type Igu N-terminally tagged with GFP [green box]. [J] Diagrammatic representation of C-terminally truncated Igu, corresponding to the iguts294e mutation, N-terminally tagged with GFP [green box]. [B–D] Ventral views of 22 somite-stage embryos. [E–G] Lateral views of 24 hpf embryos.
Subcellular distribution of the Igu protein is regulated by PKA activity

Using whole mount RNA in situ hybridization, we found that igu mRNA is maternally deposited and abundant in newly fertilized eggs during cleavage (Fig. 8A). At early and mid-somitogenesis stages, as well as at 24 hpf, we observed an essentially ubiquitous and homogeneous distribution of igu mRNA (Fig. 8B,C; data not shown), suggesting that the gene is not transcriptionally regulated by Hh activity. To analyze the subcellular distribution of the Igu protein, we made use of the chimeric cDNA encoding the functional GFP::Igu fusion described above [Fig. 6I]. Due to the relatively deep location within the embryo of Hh-responding cells, these particular cell populations could not easily be imaged to study the subcellular distribution of the GFP::Igu protein. Instead, we selected cells of the paraxial mesoderm for this assay, as they represent a large, comparatively flat, and more accessible field of cells. Moreover, although not normally exposed to Hh signaling during early somitogenesis, these cells retain the capacity to respond to ectopic activation of the pathway (Concordet et al. 1996; Hammerschmidt et al. 1996; Blagden et al. 1997; Du et al. 1997; Wolff et al. 2003). In such uninduced somitic cells, we found the GFP::Igu protein to be distributed...
Discussion

The most striking feature of the igu mutant phenotype is the paradoxical mix of depletion and ectopic differentiation of Hh-dependent cell types within the embryonic myotome. The reduction in numbers of SSFs and MPs in the anteriormost somites is indicative of an initial attenuation of Hh pathway activity in the paraxial mesoderm, an effect manifested in the reduced levels of ptc1 transcript that accumulate in the adaxial cells of early somitogenesis stage mutant embryos. This effect can be understood as reflecting a change in the balance of Gli2_{act} and Gli2_{rep} activities in favor of the latter; in support of this view, we show that removal of Gli1 from igu mutant embryos results in the complete elimination of MPs and SSFs [as well as MFFs], indicating that the levels of Gli2_{act}, which can normally completely compensate for loss of Gli1 activity in the myotome [Wolff et al. 2003], are insufficient to mediate even the lowest threshold response to Hh signaling in igu mutants. The insensitivity of igu mutants to blockade of PKA activity argues that igu acts downstream of the phosporegulation of the Gli proteins.

Despite this initial attenuation of pathway activity, all three Hh-dependent muscle cell types can nevertheless differentiate in igu mutant embryos, the late differentiating MFFs actually increasing in number, even in the absence of Shh or Smo function. This ligand independence argues against the possibility that the ectopic induction of MFFs is caused by a spreading of signal across the paraxial mesoderm, as might be predicted to occur, due to the initial low-level expression of the Hh receptor Ptc1 [see Wolff et al. 2003]. Rather, in light of the igu;_dtr phenotype, we suggest that loss of igu function also causes the low-level constitutive activation of Gli1. Initially, this activity is sufficient to compensate partially for the altered balance of Gli2_{act}/Gli2_{rep} activity, thereby accounting for the partial rescue of both MPs and SSFs in the absence of Smo activity. However, as embryogenesis proceeds, transcription of gli1, which like ptc1, is an immediate target of Hh activity in vertebrates, becomes up-regulated by the constitutive Gli1 activity [Supplementary Fig. 3], serving to amplify the effect and causing the expansion of the population of late-induced MFFs beyond the normal domain of Hh signaling.

A comparable expansion of MFFs has previously been shown to occur when Su[fu] activity is depleted. Moreover, we found that this effect is principally mediated by the deregulation of Gli1 activity, implying that at least in this context, Su[fu] acts as a negative regulator of Gli1 [Wolff et al. 2003]. Studies of Su[fu] activity in tissue culture have suggested that it acts in two ways, both to sequester Gli proteins in the cytoplasm [Ding et al. 1999; Kogerman et al. 1999] and to inhibit their transcriptional activating activity in the nucleus [Cheng and Bishop 2002]. In each case, Su[fu] is thought to mediate this effect by binding directly to the Glis, and specific domains have been defined in both Su[fu] and Gli1 that mediate their physical interaction. Thus, the effects of Su[fu] overexpression in igu mutants are entirely consistent with the notion that deregulated Gli1 is responsible for the induction of Hh-dependent muscle cell types in these embryos.

The phenotypic similarity and synergistic interaction between igu mutants and Su[fu] depletion and overexpression could imply a mechanistic similarity between igu and Su[fu] function. Moreover, we have shown that, like Su[fu], the Igu protein localizes to the cytoplasm, but translocates to the nucleus in response to the same signal that activates Gli activity. This translocation could be driven by an interaction between Igu and the Gli proteins, as seems to be the case for Su[fu]; according to this scenario, the nuclear-localized Igu might function by modulating nuclear Gli activity. Alternatively, Igu might itself regulate the nuclear-cytoplasmic shuttling of the Glis. Consistent with this notion, we find that like Su[fu] morphants, igu enhances the dominant effect of yot heterozygotes, suggesting an increased nuclear accumulation of the constitutively active Gli2_{rep} encoded by the yot mutant allele. In this regard, it is striking that the C-terminally truncated form of Igu is constitutively localized to the nucleus. Although this could imply a possible mechanism for the increased activity of both Gli1 as well as the Gli2_{rep} in igu mutants, the fact that the igu mutant alleles are completely recessive, and that misexpression of a truncated form of Igu has no effect on muscle specification argues against this. Furthermore, we have shown that the MO-mediated inhibition of Igu expression has identical phenotypic consequences to the mutant alleles that encode truncated forms of the protein, implying that the increased activities of Gli1 and Gli2_{rep} are independent of the aberrant nuclear localization of the mutant proteins.

As with Su[fu], the cytoplasmic retention of Igu may reflect its physical interaction with one or more Gli proteins. Such an interaction could be direct or via an intermediary, perhaps Su[fu] itself. The molecular structure of the Igu protein is certainly consistent with these possibilities, the single zinc-finger and the coiled-coil domains both capable of mediating protein–protein interactions; however, given the properties of the truncated protein, we would predict that any such GlI interaction would be mediated by the coiled-coil domain. Alternatively, Igu distribution in uninduced cells may be controlled primarily by the NES, which is deleted in the C-terminally truncated form of the protein. Unraveling the details of interactions between Igu and its potential partners, and the requirements for the NLS and NES,
will be essential in elucidating the basis of its contrasting effects on Gli1 and Gli2 activity.

The Hh pathway is remarkable, in that most of its known components to date have been identified through the systematic analyses of mutations in Drosophila. Despite this scenario, recent biochemical, as well as genetic studies, have led to the isolation of a number of new proteins whose inactivity disrupts Hh signaling in the mammalian embryo (Chuang and McMahon 1999; Eggenschwiler et al. 2001; Chuang et al. 2003; Huangfu et al. 2003). There is no evidence to date of a role for the orthologs of any of these proteins in regulating Hh signaling in the fly, raising the possibility that they constitute a set of dedicated elements that are required for Hh signal transduction exclusively in vertebrates. Igu represents a new and obligate member of the Hh pathway in the vertebrate embryo whose activity appears to be central to the regulation of the ultimate step in the signaling process, the nuclear access of Gli. We note that a previously uncharacterized gene, CG13617, in the annotated Drosophila genome appears to be a likely ortholog of igu. Whether or not the product of this gene is similarly involved in regulating the nuclear accumulation of Ci remains to be determined.

**Materials and methods**

**Zebrafish strains and genetics**

The dtr<sup>-209</sup>, igu<sup>12204e</sup>, syu<sup>704</sup>, syu<sup>4</sup>, yot<sup>119</sup>, and smu<sup>hi1640</sup> alleles have been previously described [Karlstrom et al. 1996; Schuette et al. 1998; Chen et al. 2001]; the igu<sup>h33</sup> allele was induced byENU and isolated in an F<sub>3</sub> lethal screen at the Center for Developmental Genetics. Because all three igu alleles exhibit similar phenotypic strengths, we have performed all of our functional analyses with igu<sup>12204e</sup>, unless otherwise mentioned. Double heterozygotes for igu<sup>12204e</sup> and syu<sup>704</sup>, syu<sup>4</sup>, yot<sup>119</sup>, and dtr<sup>-209</sup> were generated using standard genetic crosses. The syu<sup>igu</sup> and smu<sup>igu</sup> double mutant embryos were distinguished by their igu-like morphology and pattern of muscle cells in the myotome and the lack of shh and smo expression, respectively, at 24 hpf. Because both dtr and igu genes map to LG6, crosses between heterozygous igu/dtr fish produced dtr, igu double homozygotes as well as dtr, igu/+, and dtr/+, igu mutant embryos resulting from recombination events between the two loci. Whereas dtr, igu/+ embryos were indistinguishable from dtr homozygotes, dtr, igu and dtr/+, igu mutants could be differentiated by their distinct effects on muscle-cell specification. The identity of dtr/+, igu mutants was confirmed by analyzing embryos that derived from crosses between igu/dtr and igu/+ fish. The severity of the myotomal phenotype of yot/igu double-mutant embryos was indistinguishable from yot homozygotes obtained from crosses of yot/+igu/+ transheterozygous fish with the numbers of igu-like embryos always short of the expected Mendelian ratio, indicating that yot is epistatic to igu. yot/+igu embryos were obtained by crossing yot/+igu transheterozygote fish with igu/+ heterozygotes.

**Positional cloning of the igu locus**

Igu<sup>12204e</sup> heterozygous fish were crossed to the polymorphic wild-type strain WIK (L11) to produce a reference-mapping cross. A chromosomal walk was initiated by using the closely linked marker z6624 to isolate PAC/BAC clones in the region. Genomic libraries were screened by hybridization of <sup>32</sup>P-labeled DNA fragments to array filters [C. Amemiya PAC library, RZPD, and CHORI-211 BAC library, BACPAC Resources] or by PCR of pooled clones [C. Amemiya PAC library, RZPD] by use of standard methods. Isolated BAC and PAC clones were sequenced and annotated by the Wellcome Trust Sanger Center as part of their ongoing zebrafish genome sequencing project [http://www.sanger.ac.uk/Projects/D_rerio]. A full-length wild-type dzip1 cDNA clone was identified from the zebrafish EST database (and obtained from the RZPD). Full-length dzip1 cDNA clones were amplified by RT-PCR from embryos homozygous for the three different mutant alleles of igu, and directly sequenced to determine the positions of the lesions. Whereas the igu<sup>12204e</sup> allele harbors a premature stop codon in the cDNA as a consequence of nucleotide substitution, igu<sup>12204e</sup> contains a small deletion and igu<sup>h33</sup> a small insertion, respectively, that result in translational frame shifts and introduction of premature stop codons [see Supplementary Figs. 1, 2]. The GenBank accession number for the sequence of the igu cDNA is AY551927.

**Antisense igu MOs**

We utilized three different translation start-site and two splice-site targeted antisense MOs to knock down function of the IgU protein.

Their sequences are as follows: Start MO1, 5'-ACGGTTGTCTAAAAATGGCATCTTGC-3'; Start MO2, 5'-TGCGATCTTGC GTGTTTTGTGAAGT-3'; Start MO3, 5'-CTTAATGTGATCGGACGGCCG-3'; Splice MO1, 5'-GTACAGACCTTGTGGTAAATGAGCAC-3'; Splice MO2, 5'-CAGATTGAACCTCCTCATATGCGAAT-3'.

The start MOs did not produce any effects when injected into wild-type embryos, nor were they able to modify the igu mutant phenotype, indicating that they are incapable of inhibiting igu mRNA translation. The splice MOs were used at a concentration of 1 mM [0.5 mM when they were used in combination], and 3 nL of the MO solution was injected into each fertilized egg.

**Embryo injections, in situ hybridization, and antibody labeling**

Injection of DNA constructs and in vitro-synthesized mRNA, in situ hybridization, and antibody labeling were done following routine procedures. Probes for forkhead domain 4 (fkd4, Odenthaler and Nusslein-Volhard 1998), gli1 [Karlstrom et al. 2003], and ptc1 [Concordet et al. 1996], the slow myosin heavy chain [MyHC] antibody mAbE59, anti-Eng antibody mAb 4D9, anti-Prox1 antibodies, MOs against zebrafish Saffu, and gli2 [Wolff et al. 2003, and references therein] as well as capped mRNA expression constructs for shh [Krauss et al. 1993], dnpka, cpka [Conder et al. 1996], and β-galactosidase [Schilling et al. 1999] have all been described previously. A full-length zebrafish Saffu cDNA [Wolff et al. 2003] was subcloned into the SP64T expression vector. For expressing N-terminal GFP-tagged full-length wild-type and the igu<sup>12204e</sup>-specific mutant version of the IgU protein, the respective cDNAs were fused in frame with GFP in the pCS2 vector provided by J. Miller (Howard Hughes Medical Institute, University of Washington Medical School, Seattle, WA). Approximately 3 nL of 0.5 mg/mL of synthetic-capped mRNA derived from in vitro transcription reactions of the various constructs were injected into each embryo. Anti-GFP antibodies [Abcam] were used for amplification of the GFP signal from the tagged IgU proteins, and the preparations were...
counterstained with the DNA-binding dye ToPro-3 to highlight the nuclei. Cyclopamine samples were purchased from Toronto Research Chemicals and used at a concentration of 100 µM as described previously [Wolff et al. 2003].

Acknowledgments

We thank F. Wilson and L. Gleadall for help with fish husbandry; F. van Bebber and S. Glaser for assistance with genetic mapping; C. Davidson, S. Geiger-Rudolph, B.C. Lee, and S.Y. Tay for excellent technical assistance; N. Hopkins for smu mutant fish; the Sanger Institute zebrafish genome sequencing consortium for sequencing and annotation of genomic clones; F. Stockdale, S. Tomarev, and the Developmental Studies Hybridoma Bank for antibodies; J. Miller and R. Karlstrom for plasmids; C. Smythe for pointing out the NES; F. Madejsperacher for comments on drafts of the manuscript; and C. Nüsslein-Volhard for her interest and support. This work was funded by a Welcome Trust program grant [P.W.L.], the Max-Planck Institut für Entwicklungsbiologie and the German Human Genome Project [P.H. and R.G.], the Deutsche Forschungsgemeinschaft [C.W.], and the Institute of Molecular and Cell Biology and the Agency for Science, Technology, and Research, Singapore [S.R.]. Confocal microscope facilities were funded by the Yorkshire Cancer Research; the Institute of Molecular and Cell Biology; and the Agency for Science, Technology, and Research. We dedicate this work to the memory of our late friend and colleague, P. Haffter.

References

Blagden, C., Currie, P.D., Ingham, P.W., and Hughes, S.M. 1997. Notochord induction of zebrafish slow muscle is mediated by Sonic Hedgehog. Genes & Dev. 11: 2163–2175.

Brand, M., Heisenberg, C.P., Warga, R.M., Pellegri, F., Karlstrom, R.O., Beuchle, D., Pickler, A., Jiang, Y.J., Furutani-Seiki, M., van Eeden, F.J., et al. 1996. Mutations affecting development of the midline and general body shape during zebrafish embryogenesis. Development 123: 129–142.

Buttitta, L., Mo, R., Hui, C.C., and Fan, C.M. 2003. Interplays of Gli2 and Gli3 and their requirement in mediating Shh-dependent sclerotome induction. Development 130: 6233–6243.

Caspy, T., Garcia-Garcia, M.J., Huangfu, D., Eggschwiler, J.T., Wyler, M.R., Rakeman, A.S., Alcorn, H.L., and Anderson, K.V. 2002. Mouse Displaced homolog1 is required for long-range, but not juxtacrine, Hh signaling. Curr. Biol. 12: 1628–1632.

Chen, W., Burgess, S., and Hopkins, N. 2001. Analysis of the zebrafish smoothend mutant reveals conserved and divergent functions of Hedgehog activity. Development 128: 2385–2396.

Cheng, S.Y. and Bishop, J.M. 2002. Suppressor of Fused represses Gli-mediated transcription by recruiting the SAP18–msin3 corepressor complex. Proc. Natl. Acad. Sci. 99: 5442–5447.

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

Chuang, P.T. and McMahon, A.P. 1999. Vertebrate Hedgehog signaling modulated by induction of a Hedgehog-binding protein. Nature 397: 617–621.

Chuang, P.T., Kawcak, T., and McMahon, A.P. 2003. Feedback control of mammalian Hedgehog signaling by the Hedgehog-binding protein, Hip1, modulates Fgf signaling during branching morphogenesis of the lung. Genes & Dev. 17: 342–347.

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

Currie, P.D. and Ingham, P.W. 1996. Induction of a specific muscle cell-type by a Hedgehog-like protein in zebrafish. Nature 382: 452–455.

Ding, Q., Fukami, S., Meng, X., Nishizaki, Y., Zhang, X., Sasaki, H., Dlugosz, A., Nakafuku, M., and Hui, C. 1999. Mouse Suppressor of Fused is a negative regulator of Sonic Hedgehog signaling and alters the subcellular distribution of Gli1. Curr. Biol. 9: 1119–1122.

Du, S.J., Devoto, S.H., Westerfield, M., and Moon, R.T. 1997. Positive and negative regulation of muscle cell identity by members of the hedgehog and TGFβ gene families. J. Cell Biol. 139: 145–156.

Eggschwiler, J.T., Espinoza, E., and Anderson, K.V. 2001. Rab23 is an essential negative regulator of the mouse Sonic Hedgehog signaling pathway. Nature 412: 194–198.

Goodrich, L.V., Milenkovic, L., Higgins, K.M., and Scott, M.P. 1997. Altered neural cell fates and medulloblastoma in mouse patched mutants. Science. 277: 1109–1113

Hammerschmidt, M., Bitgood, M.J., and McMahon, A.P. 1996. Protein kinase A is a common negative regulator of Hedgehog signaling in the vertebrate embryo. Genes & Dev. 10: 647–658.

Huangfu, D., Liu, A., Rakeman, A.S., Murcia, N.S., Niswander, L., and Anderson, K.V. 2003. Hedgehog signaling in the mouse requires intralagellar transport proteins. Nature 426: 83–87.

Ingham, P.W. and McMahon, A.P. 2001. Hedgehog signaling in animal development: Paradigms and principles. Genes & Dev. 15: 3059–3087.

Kalderon, D. 2004. Hedgehog signaling: Costal-2 bridges the transduction gap. Curr. Biol. 14: R67–R69.

Karlstrom, R.O., Trowe, T., Klostermann, S., Baier, H., Brand, M., Crawford, A.D., Grunewald, B., Haffter, P., Hoffmann, H., Meyer, S.U., et al. 1996. Zebrafish mutations affecting retinotectal axon pathfinding. Development 123: 437–438.

Karlstrom, R.O., Talbot, W.S., and Schier, A.F. 1999. Comparative synteny cloning of zebrafish you-too: Mutations in the Hedgehog target gli2 affect ventral forebrain patterning. Genes & Dev. 13: 388–393.

Kawakami, T., Kawcak, T., Li, Y.J., Zhang, W., Hu, Y., and Chuang, P.T. 2002. Mouse displaced mutants fail to distribute hedgehog proteins and are defective in hedgehog signaling. Development 129: 5753–5765.

Kogerman, P., Grimm, T., Kogerman, L., Krause, D., Unden, A.B., Sandstedt, B., Toftgard, R., and Zaphiropoulos. 1999. Mammalian Suppressor of Fused modulates nuclear-cytoplasmic shuttling of Gli-1. Nat. Cell Biol. 1: 312–319.

Kondoh, H., Uchikawa, M., Yoda, H., Takeda, H., Furutani-Seiki, M., and Karlstrom, R.O. 2000. Zebrafish mutations in Gli-mediated Hedgehog signaling lead to lens transdifferentiation from the adenosynophysis anlage. Mech. Dev.
Krauss, S., Concordet, J.-P., and Ingham, P.W. 1993. A functionally conserved homologue of the Drosophila segment polarity gene hedgehog is expressed in tissues with polarizing activity in zebrafish embryos. Cell 75: 1431–1444.

Ma, Y., Erkner, A., Gong, R., Yao, S., Taipale, J., Basler, K., and Beachy, P.A. 2002. Hedgehog-mediated patterning of the mammalian embryo requires transporter-like function of dispatched. Cell 111: 63–75.

Matise, M.P., Epsteinm, D.J., Park, H.L., Platt, K.A., and Joyner, A.L. 1998. Gli2 is required for induction of floor plate and adjacent cells, but not most ventral neurons in the mouse central nervous system. Development 125: 2759–2770.

McMahon, A.P., Ingham, P.W., and Tabin, C. 2003. The developmental roles and clinical significance of Hedgehog signaling. Curr. Topics Dev. Biol. 53: 1–114.

Mo, R., Freer, A.M., Zinyk, D.L., Crackower, M.A., Michaud, J., Heng, H.H., Chik, K.W., Shi, X.M., Tsui, L.C., Cheng, S.H., et al. 1997. Specific and redundant functions of Gli2 and Gli3 zinc finger genes in skeletal patterning and development. Development 124: 113–123.

Moore, F.L., Jaruzelska, J., Fox, M.S., Urano, J., Firpo, M.T., Turek, P.J., Dorfman, D.M., and Pera, R.A. 2003. Human Pumilio-2 is expressed in embryonic stem cells and germ cells and interacts with DAZ [Deleted in Azoospermia] and DAZ-like proteins. Proc. Natl. Acad. Sci. 100: 538–543.

Mozyama, J., Milenkovic, L., Iwama, M., Shikata, Y., Scott, M.P., and Hui, C.C. 2003. Differential requirement for Gli2 and Gli3 in ventral neural cell fate specification. Dev. Biol. 259: 150–161.

Nakano, Y., Kim, R., Kawakami, A., Roy, S., Schier, A.F., and Ingham, P.W. 2004. Inactivation of dispatched1 by the chameleon mutation disrupts Hedgehog signaling in the zebrafish embryo. Dev. Biol. 269: 381–392.

Odenthal, J. and Nüsslein-Volhard, C. 1998. fork head domain genes in zebrafish. Dev. Genes Evol. 208: 245–258.

Odenthal, J., van Eeden, F.J., Haffter, P., Ingham, P.W., and Nüsslein-Volhard, C. 2000. Two distinct cell populations in the floor plate of the zebrafish are induced by different pathways. Dev. Biol. 219: 350–363.

Park, H.L. and Bai, C. 2000. Mouse Gli1 mutants are viable but have defects in SHH signaling in combination with a Gli2 mutation. Development 127: 1593–1605.

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

Schilling, T., Concordet, J.-P., and Ingham, P.W. 1999. Regulation of left–right asymmetries in the zebrafish by Shh and BMP4. Dev. Biol. 210: 277–287.

van Eeden, F.J., Granato, M., Schach, U., Brand, M., Furutani-Seiki, M., Haffter, P., Hammerschmidt, M., Heisenberg, C.P., Jiang, Y.J., Kane, D.A., et al. 1996. Mutations affecting somite formation and patterning in the zebrafish, Danio rerio. Development 123: 153–164.

Varga, Z.M., Amores, A., Lewis, K.E., Yan, Y.L., Postlethwait, J.H., Eisen, J.S., and Westerfield, M. 2001. Zebrafish smoothened functions in ventral neural tube specification and axon tract formation. Development 128: 3497–3509.

Wolff, C., Roy, S., and Ingham, P.W. 2003. Multiple muscle cell identities induced by distinct levels and timing of Hedgehog activity in the zebrafish embryo. Curr. Biol. 13: 1169–1181.

Zhang, X.M., Ramalho-Santos, M., and McMahon, A.P. 2001. Smoothened mutants reveal redundant roles for Shh and Ihh signaling including regulation of L/R asymmetry by the mouse node. Cell 105: 781–792.
**iguana** encodes a novel zinc-finger protein with coiled-coil domains essential for Hedgehog signal transduction in the zebrafish embryo

Christian Wolff, Sudipto Roy, Katharine E. Lewis, et al.

*Genes Dev.* 2004, 18: Access the most recent version at doi:10.1101/gad.296004

---

**Supplemental Material**  
http://genesdev.cshlp.org/content/suppl/2004/06/16/296004.DC1

**References**  
This article cites 39 articles, 19 of which can be accessed free at:  
http://genesdev.cshlp.org/content/18/13/1565.full.html#ref-list-1

**License**

**Email Alerting Service**  
Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or click here.