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

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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 (Gli_{act}) and repressor (Gli_{rep}) 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 Smoothed 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.

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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).

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Understanding how the same Hh signal can elicit multiple cellular responses in this way requires a detailed knowledge of the mechanism by which the signal is transduced. Much of what is currently known about the Hh-signaling pathway is based upon its genetic analysis in *Drosophila* (for review, see Ingham and McMahon 2001). Hh signal, released by the Dispatched (Disp) protein from secreting cells, binds to the Disp-related receptor protein Patched (Ptc) in responding cells, thereby relieving the Ptc-mediated inhibition of Smoothened (Smo), 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 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 *Gli1*, *Gli2*, and *Gli3*, 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 Eeden 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), *gli1* (Karlstrom et al. 2003), and *gli2* (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 *gli1* (*dtr*) and *gli2* (*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 ap-

proach, 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-

Figure 1. Aberrant Hh signaling and mis-specification of muscle cell-types in *igu* embryos. (A) *ptc1* expression in adaxial cells (arrows) and neural plate (asterisk) of a wild-type embryo. (B) *ptc1* expression in a similar stage *igu* mutant embryo. Note the reduction in expression in adaxial cells (small arrows) and neural plate (asterisk) and ectopic expression in paraxial mesoderm (long arrows). (C) *ptc1* expression in the myotome of a wild-type embryo. (D) Expansion in *ptc1* expression in the myotome of an *igu* mutant embryo. (E) Pattern of SSFs in the anteriormost somites of a wild-type embryo labeled with anti-slow MyHC antibodies. (F) Reduction in SSFs in the anteriormost somites of an *igu* mutant embryo labeled with anti-slow MyHC antibodies. (G) MPs (long arrows) and MFFs (short arrows) in the medial myotome of a wild-type embryo labeled with anti-Eng antibodies. (H) MPs (long arrows) and MFFs (short arrows) in the medial myotome of an *igu* mutant embryo labeled with anti-Eng antibodies. (I) MPs and MFFs in the medial myotome of a wild-type embryo labeled with anti-Eng (red) and anti-Prox1 (green) antibodies. Prox1 is expressed in all slow-twitch fibers, SSFs, and MPs. (J) MPs and MFFs in the medial myotome of an *igu* mutant embryo labeled with anti-Eng (red) and anti-Prox1 (green) antibodies. (A,B) Dorsal views of 1–2 so-

mite stage. (C–J) Lateral views of 24 hpf stage embryos. Embryos depicted in this and all subsequent figures are oriented with anterior to the left, unless mentioned otherwise.

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 *Igu* acts downstream of Shh and Smo 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 cy-

toplasmic 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 supernumerary 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 effects on Gli1 and Gli2 activities

To elucidate the effects of *igu* on Gli activity further, we manipulated the expression of Gli1 or Gli2 indepen-

Figure 2. *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 *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 *igu^{ts294e}* 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 *igu^{tm79a}* mutant embryo. (G) Complete absence of Eng expression from the myotome of a *syu* mutant embryo. (H) *igu*-like pattern of Eng expression in the myotome of a *syu;igu^{ts294e}* double-mutant embryo. MPs (long arrow) and MFFs (short arrow) are indicated. (I) Complete absence of Eng expression from the myotome of a *smu* mutant embryo. (J) *igu*-like pattern of Eng expression in the myotome of a *smu igu^{ts294e}* 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 *igu^{tm79a}* mutant embryo when treated with cyclopamine ($n = 7$). All panels show lateral views of 24 hpf stage embryos.

dently in *igu* mutant embryos. In the presence of wild-type *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 *igu* and the Gli1 loss-of-function mutant *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 *igu* embryos that are hetero-

Figure 3. The *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 *igu* mutant embryo ($n = 17/21$). (C) Ectopic expression of *ptc1* in a wild-type embryo in response to dnPKA overexpression ($n = 34/34$, cf. Fig. 1A). (D) The pattern of *ptc1* expression in an *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.

Figure 4. Igu function is essential for proper Gli activity. (A) Slow muscle fibers in the myotome corresponding to somites 5–10 of a wild-type embryo revealed by histochemical staining with antibodies to slow MyHC. MP cells in the medial region of the myotome are indicated (arrows). (B) Similarly labeled slow fibers of an *igu* mutant embryo. (C) Slow fibers are almost completely absent from the myotome of an *igu;dtr* double-mutant embryo. SSF, MP, and MFF pattern in *dtr* mutants themselves are indistinguishable from wild-type embryos (Wolff et al. 2003). (D) An *igu;dtr* double-mutant embryo labeled with anti-Eng antibodies showing complete lack of Eng expression from the myotome. (E) An *igu;dtr/+* embryo stained with antibodies for slow MyHC, showing a reduction in numbers of SSFs and the absence of the medial bundle of MP fibers. (F) Complete absence of MP-specific high-level Eng expression from the myotome of an *igu;dtr/+* embryo. Residual expression of Eng in some MFFs is indicated (arrows). (G) A *yot/+* heterozygous embryo labeled with anti-slow MyHC antibodies showing an essentially wild-type like pattern of slow muscle fibers, but with the sporadic loss of the medial bundle of MP cells (arrow). (H) Complete absence of all slow muscle fibers from the myotome of an *igu;yot/+* embryo. (I) A wild-type embryo injected with *gli2* MOs and labeled with anti-Eng (red) and anti-Prox1 (green) antibodies, showing mislocation of MP-like cells on the myotomal surface (arrows, $n = 14/21$). (J) The same embryo showing complete lack of MP cells in the medial region of the myotome and an increase in the numbers of MFFs (cf. Fig. 1I). (K) An *igu* embryo injected with *gli2* MOs and labeled with anti-Eng (red) and anti-Prox1 (green) showing a reduction in the numbers of SSFs and mislocation of MP-like cells on the myotomal surface (arrows, $n = 9/12$). (L) The same embryo showing complete lack of MP cells in the medial region of the myotome and a further increase in Eng expression in the MFFs (cf. Fig. 1J). All panels depict lateral views of 24 hpf stage embryos.

zygous for *dtr* (*igu; dtr/+* embryos, Fig. 4E,F). It follows that Gli2 function is unable to compensate for the reduction 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. 4I–L). 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 Gli_{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. 4G; 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 leucine-rich motif matching the nuclear export signal (NES) consensus sequence LX_{1–3}LX_{2–3}LXL (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*^{ts294e} 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

Figure 5. (A) A wild-type embryo injected with *Su(fu)* MOs exhibiting expansion in the numbers of Eng expressing MFFs (short arrows, $n = 15/26$, cf. Fig. 2D). MPs are unaffected (long arrows). (B) A similarly injected *igu* mutant embryo with a striking increase in the numbers of Eng expressing MFFs (short arrows, $n = 10/14$). MPs are unaffected (long arrows, cf. Fig. 2E,F). (C) A *Su(fu)* mRNA-injected wild-type embryo labeled with anti-slow MyHC antibodies showing normal pattern of slow muscle fibers ($n = 22/22$). (D) A similarly manipulated sibling embryo stained with anti-Eng antibodies showing normal pattern of Eng expression in the myotome ($n = 18/18$). MPs (long arrows) and MFFs (short arrows) are indicated. (E) A *Su(fu)* mRNA-injected *igu* mutant embryo labeled with anti-slow MyHC antibodies showing a dramatic loss of slow muscle fibers ($n = 10/14$, cf. Fig. 4B). (F) A similarly injected *igu* embryo showing complete absence of MPs with residual Eng expression persisting in a few MFFs (arrows, $n = 16/19$, cf. Fig. 2E).

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 *fkf4*. Note that *fkf4* 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 *fkf4* expression remains restricted to a single row of MFP cells. (D) Restoration of patches of *fkf4* 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 *igu*^{ts294e} 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 *igu*^{ts294e} 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 *igu*^{ts294e} 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.

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, in-

jection of the MOs into the *igu*^{ts294e} 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).

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Figure 7. The *igu* phenotype represents complete loss-of-function. (A) Diagrammatic representation of the first four coding exons of the *igu* gene, the relevant nucleotide (and the corresponding amino acid) sequences at the exonic junctions and the splice sites recognized by the two MOs. (B) A wild-type embryo injected with a combination of splice MO1 and MO2 showing an *igu*-like pattern of Eng expression in the myotome ($n = 22/27$, cf. Fig. 2D–F). (C) A similarly injected wild-type embryo with no effect on Eng expression pattern after treatment with cyclopamine ($n = 10/12$, cf. Fig. 2K,L).

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 de-

scribed 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

Figure 8. Subcellular localization of the Igu protein, but not the transcription of the *igu* gene, is regulated by Hh activity. (A) Pattern of maternal *igu* mRNA distribution in the animal cells of a 16-celled stage embryo. (B) Uniform pattern of zygotic *igu* mRNA distribution in the midline and somites of a 12-somite stage embryo. (C) Uniform pattern of *igu* mRNA transcription in the myotome of a 24 hpf embryo. (D) Exclusively cytoplasmic distribution of GFP-tagged wild-type Igu in the paraxial mesodermal cells of a wild-type embryo at the 2-somite stage ($n = 10/10$). This chimeric protein is able to fully substitute for wild-type Igu activity and rescue *igu* mutant embryos (Fig. 6E). (E) Predominantly nuclear accumulation of the GFP-tagged *igu*^{ts294e} mutant form of Igu ($n = 10/10$). (F) Nuclear translocation of GFP-tagged wild-type Igu in response to inactivation of PKA activity ($n = 12/15$). (G) Nuclear accumulation of GFP-tagged *igu*^{ts294e} mutant form of Igu remains unaltered on inactivation of PKA activity ($n = 10/10$). (H) Nuclear accumulation of GFP-tagged *igu*^{ts294e} mutant form of Igu remains unaltered on overexpression of Su(fu) ($n = 10/10$). (I–M) Superimposition of the GFP images shown in D–H with those highlighting the nuclei in the same preparations after staining with TOPRO-3. A, B, and D–M are dorsal views; C is oriented laterally.

exclusively in the cytoplasm (Fig. 8D,I). Strikingly, however, there is a substantial translocation of the protein into the nucleus in embryos simultaneously injected with dnPKA mRNA (Fig. 8F,K). In contrast, the GFP-tagged truncated form of the Igu protein that retains the putative NLS but lacks the putative NES (mimicking that predicted to be encoded by the mutant allele *igu^{ts294e}*; Fig. 6J) accumulated exclusively in the nuclei of expressing cells (Fig. 8E,J), a distribution that was unaffected by blockade of PKA activity (Fig. 8G,L) or by the overexpression of Su(fu) (Fig. 8H,M).

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 Gli_{2^{act}} and Gli_{2^{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 Gli_{2^{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 phosphoregulation 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 Gli_{2^{act}}/Gli_{2^{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 Gli_{2^{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 Gli_{2^{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 Gli_{2^{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; Eggeneschwiler 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^{ts269}*, *igu^{ts294e}*, *igu^{tm79a}*, *syu^{t4}*, *yot^{ty119}*, and *smu^{hi1640}* alleles have been previously described (Karlstrom et al. 1996; Schauerte et al. 1998; Chen et al. 2001); the *igu^{b33}* allele was induced by ENU and isolated in an F3 lethal screen at the Center for Developmental Genetics. Because all three *igu* alleles exhibit similar phenotypic strengths, we have performed all of our functional analysis with *igu^{ts294e}*, unless otherwise mentioned. Double heterozygotes for *igu^{ts294e}* and *syu^{t4}*, *smu^{hi1640}*, *yot^{ty119}*, and *dtr^{ts269}* were generated using standard genetic crosses. The *syu;igu* and *smu;igu* 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^{ts294e} heterozygous fish were crossed to the polymorphic wild-type strain WIK (L11) to produce a reference-mapping cross that was used for linkage analysis. 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 ³²P-labeled DNA fragments to arrayed 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_zerio). 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^{ts294e}* allele harbors a premature stop codon in the cDNA as a consequence of nucleotide substitution, *igu^{tm79a}* contains a small deletion and *igu^{b33}* 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'-ACGTTGTCA TAAATGGCATCTTGC-3'; Start MO2, 5'-TGGCATCTTGC GTGTTTTGTGAAGT-3'; Start MO3, 5'-CTTAATGTATGAT CGGAGCGGCGCG-3'; Splice MO1, 5'-GTACAGACCTTGT GGTAATTGGCAC-3'; Splice MO2, 5'-CAGATTGAAC TCATGTCTGAAT-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* (*fkf4*; Odenthal and Nusslein-Volhard 1998), *gli1* (Karlstrom et al. 2003), and *ptc1* (Concordet et al. 1996), the slow myosin heavy chain (MyHC) antibody mAbF59, anti-Eng antibody mAb 4D9, anti-Prox1 antibodies, MOs against zebrafish *Su(fu)*, and *gli2* (Wolff et al. 2003, and references therein) as well as capped mRNA expression constructs for *shh* (Krauss et al. 1993), *dnpka*, *cpka* (Concordet et al. 1996), and β -galactosidase (Schilling et al. 1999) have all been described previously. A full-length zebrafish *Su(fu)* 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^{ts294e}*-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 μ M as described previously (Wolff et al. 2003).

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

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