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Gli2 functions in FGF signaling during antero-posterior patterning

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SUMMARY

Patterning along the anteroposterior (A-P) axis involves the interplay of secreted and transcription factors that specify cell fates in the mesoderm and neuroectoderm. While FGF and homeodomain proteins have been shown to play different roles in posterior specification, the network coordinating their effects remains elusive. Here we have analyzed the function of Gli zinc-finger proteins in mesodermal A-P patterning. We find that Gli2 is sufficient to induce ventroposterior development, functioning in the FGF-brachyury regulatory loop. Gli2 directly induces *brachyury*, a gene required and sufficient for mesodermal development, and *Gli2* is in turn induced by FGF signaling. Moreover, the homeobox gene *Xhox3*, a critical determinant of posterior development, is also directly regulated by Gli2. Gli3, but not Gli1, has an activity similar to that of Gli2 and is expressed in ventroposterior mesoderm after *Gli2*. These findings uncover a novel function of Gli proteins, previously only known to mediate hedgehog signals, in the maintenance and patterning of the embryonic mesoderm. More generally, our results suggest a molecular basis for an integration of FGF and hedgehog inputs in Gli-expressing cells that respond to these signals.

Key words: Anteroposterior patterning, Gli1, Gli2, Gli3, FGF, Mesoderm, Sonic hedgehog, *Xenopus*

INTRODUCTION

Axis formation in vertebrate embryos requires the determination of head, trunk and tail structures. The head organizer is found in the early dorsal lip area of the young gastrula that later takes a position underlying the forebrain. In contrast, the posterior organizer includes more ventral cells of the young gastrula that converge towards the posterior dorsal midline and later form the growing tail bud. For this reason, early-ventral/later-posterior (ventroposterior) fates can be thought of as having a similar molecular basis. Indeed, the homeodomain protein Xhox3 (Evx1) is expressed ventrally in the young frog gastrula and later in posterior regions, and it is involved in directing ventroposterior fates, including tail growth (Ruiz i Altaba and Melton, 1989a,b; Ruiz i Altaba et al., 1991; Beck and Slack, 1999). Other factors such as FGFs and Hox proteins also appear to be involved in posterior development (e.g. Ruiz i Altaba and Melton, 1989b; Amaya et al., 1991; Pownall et al., 1996). Nevertheless, how different signals and gene functions are integrated in a regulatory network to orchestrate posterior development remains unclear.

We have investigated a role of Gli proteins in the early embryonic mesoderm during formation of the A-P axis. In vertebrates, there are three known *Gli* genes, which appear to act in a combinatorial fashion to mediate hedgehog (HH) signals. However, their expression patterns and discerned activities suggest a context-dependent functional divergence (Ruiz i Altaba, 1997). For example, in the neural plate of the frog embryo, *Gli1* is the only *Gli* gene expressed in midline cells and its ectopic expression mimics the floor plate-inducing effects of sonic hedgehog (SHH) (Lee et al., 1997). In mice, Gli1 can also mimic the effects of SHH (Hynes et al., 1997; Sasaki et al., 1997). *Gli2*, like *Gli1*, can be induced by SHH and can promote motoneuron development in the neural tube, partially mimicking the effects of SHH (Ruiz i Altaba, 1998). However, Gli2 can inhibit floor plate induction by Gli1, and Gli3 appears to have a general antagonistic interaction with SHH and Gli1 (Marigo et al., 1996; Lee et al., 1997; Ruiz i Altaba, 1998). In other systems, there is also evidence for a transient role of Gli2, and possibly Gli3, in the mediation of SHH signaling (Dai et al., 1999; Sasaki et al., 1999; Park et al., 2000; but see Ding et al., 1998). Nevertheless, the fact that *Gli2* and *Gli3* are often expressed at a distance from sources of HH signals in different cell groups and organs (reviewed in Ruiz i Altaba, 1999b), raises the possibility that the encoded proteins may also respond to and/or mediate signaling inputs other than those provided by HH molecules.

The effects of HH signaling can be modified by other signaling inputs. For example, in the cerebellum, SHH is a mitogen, inducing proliferation of granule cell progenitors (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999), but FGF inhibits this function (Weschler-Reya and Scott, 1999). By contrast, in the neural tube, the induction of serotonergic and dopaminergic neurons in the ventral mid- and hindbrain depends on a synergistic interaction between SHH and FGF signaling (Ye et al., 1998).

SHH also appears to interact with FGFs, WNTs and BMPs (bone morphogenic proteins) in other systems, such as the developing limb bud, somites and lungs, and during left-right axis formation (e.g. Laufer et al., 1994; Yang and Niswander, 1995; Münsterberg et al., 1995; Bellusci et al., 1997; Murtaugh et al., 1999; Meyers and Martin, 1999). The molecular bases of these context-dependent positive or negative interactions remain unknown, although recent results in somitic mesoderm show that WNT signals might regulate *Gli2* (Borycki et al., 2000) and that SHH induces the expression of *Sfrp2*, a WNT antagonist (Lee et al., 2000).

In this study, we explore the function of Gli proteins in the embryonic mesoderm and their interaction with non-HH signaling events involved in A-P patterning. We first identify a member of the *Sal* family (*SalB*) expressed in the early mesoderm as a downstream gene indirectly regulated by Gli2. We then show that *Xbra* and *Xhox3* are directly induced by Gli2, which is sufficient to promote ventroposterior development. The requirement of Gli2 is suggested by the ability of its repressor forms to inhibit endogenous mesoderm development. These and other results suggest that Gli2 (and later Gli3) functions in the specification of ventroposterior fates, acting in the FGF-brachyury pathway. Moreover, our findings suggest a molecular basis for an integration of HH and FGF signaling in multiple systems.

MATERIALS AND METHODS

Animals, explants and microinjection

Xenopus laevis albino frogs and embryos were used and reared by standard techniques. Capped synthetic RNA was injected at 2ng/10nl/embryo unless otherwise noted. eFGF RNA was injected at 50pg/embryo. In co-injection experiments, each RNA was injected at 1ng/embryo matched with the same amount of control injected RNA. *lacZ* RNA used as a lineage tracer was co-injected at ~0.2ng/embryo. For animal cap injections, the RNA was targeted to the animal-most region. Marginal zone injections were targeted to the equator. Animal caps were dissected from stage 8 embryos by standard techniques with a hair knife. Cycloheximide (CHX, 5 μM) was added at stage 7-7.5 before the mid-blastula transition and cutting the animal caps. Those treated were kept in CHX until harvested.

Gastrula cDNA library, screen, clones and RT-PCR

A stage 12 *Eco*RI-*Xho*I cDNA library in lambda ZapExpressTM was constructed (Stratagene) and screened with cDNA probes derived from gastrula embryos injected with Gli2 and with a Gli2 frameshift mutant. This mutant was created by deleting the *Bam*H1-*Sma*I fragment of pCS2-Myc-fGli2 (Brewster et al., 1998), which resulted in a complete frameshift of C-terminal sequences. Of 22,000 clones screened at low density (2000 plaques per 150mm plate), we isolated five candidate Gli2 targets, one of which is reported in this paper. Full-length and truncated *Gli1, Gli2 and Gli3* cDNAs, probes and RNAs were as described in Ruiz i Altaba (1999a). Other sense RNAs for injection were made by digesting plasmids and transcribing them as follows: *Xbra*, *Sal*I and SP6 (Smith et al., 1991); *eFGF*, *Cla*I and T7 (Isaacs et al., 1994); *XFD*, *Eco*RI and SP6 (Amaya et al., 1991); *Smad2-lacZ*, *Xba*I and SP6 (Baker and Harland, 1996); activated *activin receptor type I* (T206E), *Xba*I and SP6 (Chang et al., 1997); pCS2-*cytlacZ*, *Not*I and SP6 (Turner and Weintraub, 1994).

For RT-PCR analyses, stage 9+/10 and stage ~14 embryos and animal caps were dissected, and dorsal and ventral equatorial regions collected. cDNA made from the RNA isolated from these dissections was used for PCR with the following primer pairs: *Gli2*U, 5′CATGCAGGATAACAGCATGC3′ and *Gli2*D, 5′TCTGGCTGTACCTCTGTTGG3′; *Gsc*U, 5′ACAACTGGAAGCACTGGA3′ and *Gsc*D, 5′TCTTATTCCAGAGGAACC3′; *Msx1*U, 5′ACTGGTGTGAAGCCGTCCCT3′ and *Msx1*D, 5′TTCTCTCGGGACTCTCAGGC3′; *Xbra*U, 5′GGATCGTTATCACCTCTG3′ and *Xbra*D, 5′GTGTAGTCTGTAGCAGCA3′; *EF1α*U, 5′CAGATTGGTGCTGGATATGC3′ and *EF1α*D, 5′ACTGCCTTGATGACTCCTAG3′; *Xhox3*U, 5′ACTGTAACTCGGCAGC3′ and *Xhox3*D, 5′GGATCCGGTAGGTGGC3′; *Pintallavis*U, 5′CAACATGACCAATGTCTTGCC3′ and *Pintallavis*D, 5′GGATCCGCAGCAGTGGAAGG3′; *SalB*U, 5′ATCAATGCTGGACTTGC3′ and *SalB*D, 5′GACAGAAATCTCGTTGG3′. Degenerate primers for *Ptch* genes were as described in Takabatake et al. (1997). All reactions were carried out for 30 cycles and the results analyzed in 2% agarose gels stained with ethidium bromide.

In situ hybridization, immunocytochemistry, β-gal staining and histology

Whole-mount in situ hybridization was performed with digoxigenin-labeled single-stranded RNA probes using maleic acid buffer. Reactions yielded a purple precipitate. Embryos were either viewed before or after clearing with benzyl benzoate/benzyl alcohol. *Gli* probes were as described in Lee et al. (1997) and Ruiz i Altaba (1998); *sonic hedgehog* probes were as described in Ruiz i Altaba et al. (1995). Other antisense probes were made by digesting and transcribing plasmids as follows: *N-tubulin*, *Bam*HI and T3 (Richter et al., 1988); *Xbra*, *Stu*I and T7 (Smith et al., 1991); *SalB*, *Spe*I and T7; *XmyoD*, *Xba*I and SP6 (Hopwood et al., 1992); *Msx1*, *Eco*RI and T3 (Suzuki et al., 1997); eomesodermin, *Eco*RI and T3 (Ryan et al., 1998). Myc labeling was performed after the in situ procedure. Anti-Myc monoclonal antibodies (9E1; Santa Cruz) were used at 1/500-1/2000. Secondary peroxidase-coupled antibodies were followed by development with DAB and hydrogen peroxide to yield a red-brown precipitate. Mouse monoclonal Xen1 (Ruiz i Altaba, 1992) and rabbit polyclonal anti-HNF-3β (Ruiz i Altaba et al., 1995) antibodies were used at 1/5 and 1/8000 respectively. For β-gal staining, fixed embryos were incubated with X-gal substrate before treatment with methanol. Histology was performed on stained embryos that were dehydrated in methanol and xylene and embedded in paraplast. Sections (12-14 μm) were cut with a microtome. Embryos were photographed with color slide film using a Leica binocular microscope or a Zeiss axiophot.

RESULTS

An embryonic mesodermal marker responds to Gli2

To investigate how Glis function, we performed a differential screen for genes the expression of which can be induced by Gli2. Embryos were injected with synthetic *Gli2* mRNA into the animal pole at the one- to two-cell stage and harvested at midgastrula stages (stages ~11.5-12.5). cDNA prepared from these embryos as well as from sibling embryos injected with a frameshift Gli2 construct was used to differentially screen a gastrula (stage ~12) cDNA library. Of the several candidate clones obtained that showed specific upregulated expression in Gli2-injected embryos, one was expressed in the embryonic mesoderm (Fig. 1A; see below). This clone encodes an open reading frame (Fig. 1B) with homology to the Spalt (Sal) family of zinc-finger transcription factors (Kühnlein et al., 1994). The partial protein encoded in our cDNA (GenBank Accession number AF181559) has higher homology to the human SALL1 (Kohlhase et al., 1996) than to the previously identified frog Sal1 protein (Xsal1; Hollemann et al., 1996; Fig. 1B), raising the possibility that our cDNA derives from a

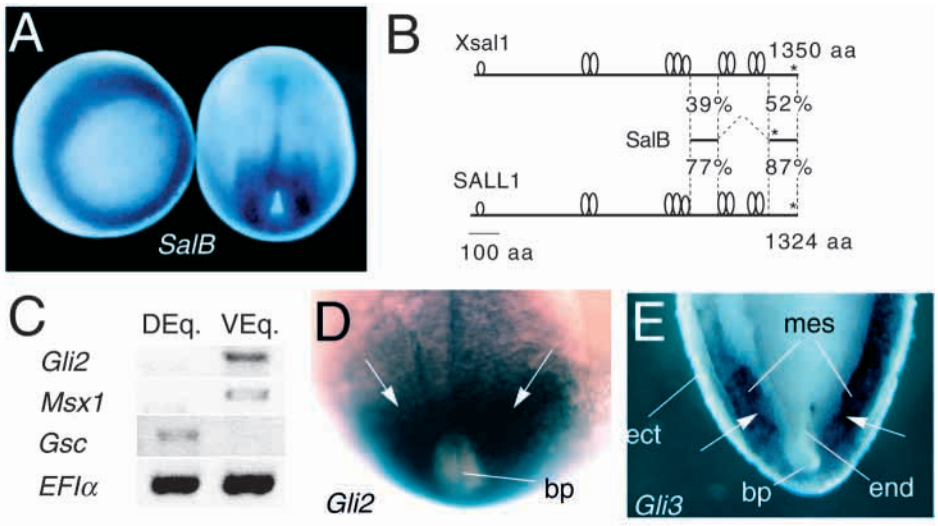

Fig. 1. Expression of *SalB*, *Gli2* and *Gli3* in mesoderm. (A) Endogenous expression of *SalB* in the marginal zone of early gastrula (stage ~10, left), and in the notochord and circumblastoporal region of late gastrula-early neurula (stage ~13, right) embryos. Weak expression is also present in the posterior neural plate. The embryo to the left shows a vegetal view and the one to the right shows a dorsoposterior view. (B) Diagram of the structure of the Xsal1 (Hollemann et al., 1996), SALL1 (Kohlhase et al., 1996) and SalB predicted proteins. Zinc fingers are denoted by vertical ellipses. Percent identities are given between homologous regions limited by broken lines. Note the lack of the last two sets of two fingers in SalB and the premature termination (*). The percent homology given for the region C-terminal to the stop codon (*) corresponds to the hypothetical ORF that would be in frame in a nonspliced version. (C) RT-PCR analyses localizing the expression of *Gli2* to the ventral equatorial region of late blastulae/early gastrulae, like *Msx1* and unlike *goosecoid* (*Gsc*). Expression of the housekeeping gene *EF1α* is used as control. (D,E) Endogenous expression (arrows) of *Gli2* (D) and *Gli3* (E) in the circumblastoporal region of late gastrula (stage ~12.5-13) embryos. (E) A filleted embryo viewed from the inside, localizing *Gli3*-expressing cells to the mesodermal layer (arrows). bp, blastopore; ect, ectoderm; end, endoderm; mes, mesoderm. In all cases anterior is towards the top except in A, left, which shows a vegetal view.

homolog of *SALL1*, a gene mutated in Towne-Brocks syndrome (Kohlhase et al., 1998). However, its structure suggests that it is a differentially spliced form that skips over the last four zinc fingers (Fig. 1B). Differentially spliced variants have also been implied from RT-PCR analyses of *Xsal1* (Hollemann et al., 1996). In both cases, the open reading frames would terminate prematurely as the sequences of the downstream exons are out of frame.

In situ analyses revealed that the identified *Sal* gene is expressed in the entire marginal zone (Fig. 1A left) and, at later stages, it is found in the circumblastoporal region and in the notochord (Fig. 1A right), recapitulating the expression of *brachyury* (*Xbra*; e.g. Fig. 2B; Smith et al., 1991). It is also expressed at very low levels in the most posterior neural plate (Fig. 1A right), which is fated to give rise to mesoderm. This expression is distinct from that of *Xsal1*, as the latter is not expressed in mesoderm, showing instead prominent expression in the neural plate and neural tube (Hollemann et al., 1996). Similarly, a mouse *Sal1* gene closely related to *Xsal1* is also expressed in the neural tube and not in the early mesoderm (Ott et al., 1996). For these reasons, we have named our form *SalB*, for its identity to the *Sal* gene and its expression patterns similar to that of *brachyury*. Given the identification of *SalB* as an upregulated gene in Gli2-injected embryos, we tested for its ectopic expression by whole-mount in situ hybridization in embryos injected with *Gli2*. *SalB* mRNA was detected at ectopic positions (Fig. 2C; stages 12-14; *n*=29/59, Myc$^+$, see below), indicating that Gli2 is sufficient to induce *SalB*. Since regulation of *SalB* by Gli2 appears to be indirect (see below), we have only used it as a marker of embryonic mesoderm.

The *Gli* genes are expressed in the embryonic mesoderm

The induction of *SalB* by Gli2 raises the possibility that *Gli* genes might be involved in mesodermal development, a process thought to be independent of HH signaling (Ruiz i Altaba et al., 1995, Ekker et al., 1995; Chiang et al., 1996). *Gli2* and *Gli3* are expressed in the early mesoderm of frog gastrulae and in the tail bud at later stages (Lee et al., 1997; Marine et al., 1997). Whereas *Gli* gene expression is not detected maternally, *Gli2* mRNA is present in blastulae (with an onset of expression before stage 9; Marine et al., 1997), *Gli3* is first expressed in early gastrulae (at stage 11.5; Marine et al., 1997) and *Gli1* is detected in the dorsal midline during gastrulation (Lee et al., 1997). RT-PCR analyses of dissected dorsal and ventral equatorial regions of late blastula/early gastrula (stage 9+/10) embryos showed that *Gli2* is not expressed in the most dorsal mesoderm, the region of the organizer (Fig. 1C). *Gli2* expression coincides with that of the ventral marker *Msx1* (Suzuki et al., 1997), and not with that of *goosecoid* (Cho et al., 1991) in the dorsal mesoderm (Fig. 1C). As control, both samples showed equivalent levels of transcripts of the housekeeping gene *EF1α* (Fig. 1C). By mid to late gastrula stages (stages ~12-13), *Gli2* and *Gli3*, like *Xbra* (not shown; Smith et al., 1991), are expressed in mesoderm of the circumblastoporal region (Fig. 1D-E), which will give rise to posterior structures, including the tail.

Gli2 induces ectopic *brachyury* expression

To test for a general role of *Gli* genes in the early mesoderm, we assayed for the response of *brachyury* (*Xbra*), a gene required and sufficient for mesodermal development (Smith et al., 1991; Cunliffe and Smith, 1992), to injected Gli proteins. The expression and levels of Myc-tagged Gli proteins was determined by immunocytochemical detection of the Myc epitopes (denoted as Myc$^+$). Myc tagging localizes the expression of Gli proteins with single-cell resolution and allows a careful correlation of their effects on the expression of the markers tested.

Injection of Gli2 induced ectopic *Xbra* expression (Fig. 2A,

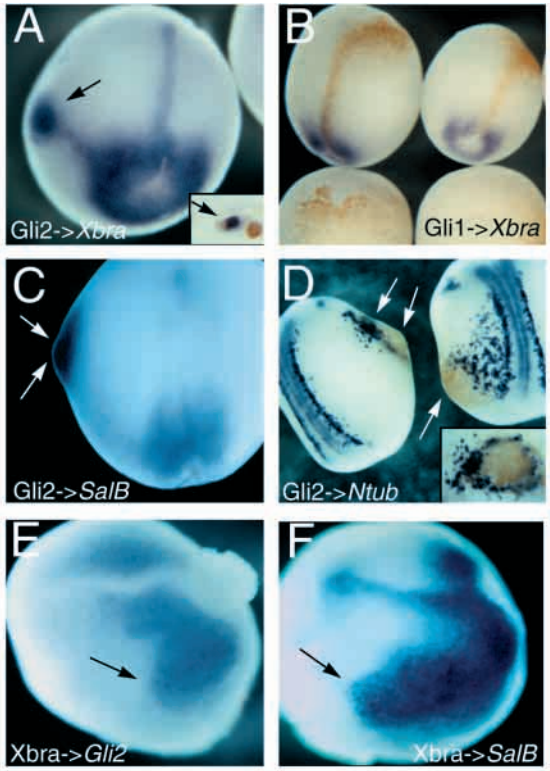

Fig. 2. Gli2 induces mesodermal and neuronal differentiation. (A,B) Ectopic expression of *Xbra* in a Gli2-injected gastrula (stage ~12.5-13) embryo (A), but not in Gli1-injected sibling embryos (B). Double-labeling with anti-Myc antibodies (A,B) reveals the position of cells expressing the injected Myc-tagged Gli proteins. The inset in (A) shows two Myc-Gli expressing cells in a histological section, of which only one also expresses *Xbra* (arrow). (C) Ectopic expression of *SalB* in a protrusion (arrows) induced by injected Gli2 in a stage ~12.5-13 embryo. (D) Induction of ectopic N-tubulin (*Ntub*) expression in Gli2-injected embryos at neurula stages (stage ~15). Note the protrusions and the separation of Myc^+/N-$tubulin^-$ cells from Myc^+/N-$tubulin^+$ cells (inset). (E,F) Induction of ectopic *Gli2* (E) and *SalB* (F) expression in late gastrula embryos by injected Xbra. In each part, arrows point to sites of ectopic gene expression. The panels show dorsal views in which anterior is towards the top, except in (E,F) in which it is towards the left. Myc labeling is brown-red and in situ hybridization signal is blue.

stage 12-14; *n*=10/31 ectopic, Myc^+). Histological sections revealed the co-localization of *Xbra* expression and Myc immunoreactivity, although not all Myc^+ cells expressed *Xbra* mRNA (Fig. 2A, inset). At earlier stages, the expression of *Xbra* (stage ~10-11, 0/13 ectopic, Myc^+; not shown) and *SalB* (stage ~10-11, *n*=0/5 ectopic, Myc^+; not shown) were not induced ectopically by injected Gli2. Gli3 induced ectopic *Xbra* expression in a minority of embryos (stage ~12-14; *n*=2/52 ectopic; not shown). Because injections of RNA into the most animal region result in the restricted localization of the encoded proteins to the animal pole, not ever reaching the marginal zone (Ruiz i Altaba et al., 1995 and not shown), it is safe to assume that Gli2 and Gli3 induced mesodermal gene expression in ectoderm. Indeed, injection of Gli2 into a single animal blastomere at the 16-32-cell stage resulted in ectopic *Xbra* expression (not shown). Control embryos did not show ectopic *Xbra* expression (not shown; *n*=0/30), and ectopic Gli1 expression in the animal pole also did not induce ectopic *Xbra* expression (Fig. 2B; stage ~12-14; *n*=0/43, Myc^+). This is consistent with the different behavior of Gli1 and Gli2/3 in most assays (reviewed in Ruiz i Altaba, 1999b).

Gli2-injected embryos showed small protrusions coincident with Myc-Gli2 expressing cells. These protrusions are also coincided with the expression of *SalB* (Fig. 2C) and *Xbra* (Fig. 2A), indicating that they contain ectopic mesodermal cells in the epidermis. Analyses of neuronal differentiation in these embryos showed that the pan-neuronal marker *N-tubulin* was induced by Gli2 as expected (Brewster et al., 1998), and expressed in Myc-labeled cells surrounding these mesodermal bumps (Fig. 2D, inset; stage ~14, *n*=14/14, Myc^+). This indicates that Gli2-expressing cells that acquire either mesodermal or neuronal fates segregate.

Gli2 induces ventroposterior development

To test for the fate of Gli2-injected cells, Gli2 was co-injected with *lacZ* mRNA into the animal pole and the injected embryos allowed to develop until tadpole stages (stages ~32-36). β-galactosidase (β-gal) activity was then revealed by X-gal staining, as a marker for cells expressing the injected material. Injection of *lacZ* alone into the animal cap region resulted in normal development of the epidermis and the normal scattering of cells inheriting the injected material (Fig. 3A, *n*=25/25). In contrast, co-injection with *Gli2* resulted in ectopic appendages that had the appearance of ectopic tails (Fig. 3B-D; *n*=41/79) often with fins (Fig. 3C-E). Gli3 also induced tail-like structures (Fig. 3H; *n*=42/109 and not shown), but Gli1 did not (*n*=0/50; not shown). The tips of the Gli2-induced tail-like structures were found to have the highest level of β-gal activity (Fig. 3B,C), suggesting that cells expressing Gli2 clustered and formed the growing tip. Histological examination of ectopic tail-like structures showed the presence of β-gal staining in both the ectodermal tip and the underlying mesoderm, consistent with the induction of mesoderm by Gli2 (Fig. 3C inset). The large embryonic endodermal cells at these stages can be easily recognized by their histological appearance. This cell type was not found within the induced tails (not shown). The induction of posterior (tail) development by Gli2 is consistent with the failure to induce anterior markers, such as eomesodermin (*n*=0/21 ectopic) at late neurula stages (stage ~20), when the latter is expressed in prechordal plate mesoderm (not shown; Ryan et al., 1998).

Analyses of the cell types induced in the ectopic tail-like structures at tadpole stages showed that axial cells are absent, as tested by the lack of *Shh* (Fig. 3E; *n*=0/15), which is normally expressed in cells of the notochord, hypochord and floor plate (Ruiz i Altaba et al., 1995; Ekker et al., 1995). Expression of *MyoD*, a marker of somites (Hopwood et al., 1992), was detected in a subset of tails and only in a small number of cells (Fig. 3F; *n*=3/10), indicating that paraxial muscle is not often included. Induced tail-like structures, thus appear to be composed of lateral and/or ventral tissues, suggesting that these have a ventral tail character, in agreement with the fate of early-ventral/later-posterior cells. In addition, this conclusion is further supported by marker and morphological analyses of Gli2-injected animal caps (see below).

Consistent with the ability of Gli2 and Gli3 to induce neurogenesis (Brewster et al., 1998), tail-like structures

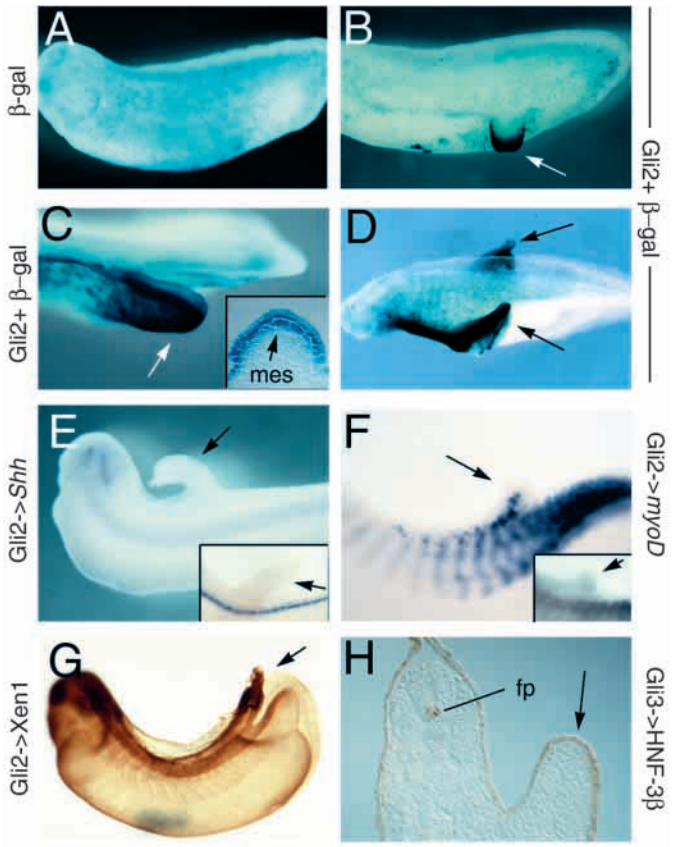

Fig. 3. Gli2 and Gli3 induce ectopic tail development. (A-D) Expression of injected *lacZ* alone in epidermis (A) does not affect the morphology of the resulting tadpole (stage ~32), whereas co-injection of *lacZ* (as a lineage marker) and *Gli2* RNAs result in the induction of tails (B-D, arrows) in stage ~34 embryos. β-gal$^+$ cells accumulate at the tail tips. The inset in C shows a histological section of an induced tail in a stage ~32 embryo showing the presence of labeled cells in both mesoderm (mes, arrow) and overlying ectoderm. The embryo in (D) received two injections into each cell at the two-cell stage and developed bilateral ectopic tails (arrows). (E) *Gli2*-injected embryos show ectopic tails (arrows) that lack *Shh*$^+$ cells and thus lack notochord, hypochord or floor plate cells. (F) Ectopic tails induced by Gli2 rarely contain *myoD*$^+$ cells, indicating that somitic tissue is not often included. (G) Xen1$^+$ neural differentiation is detected in Gli2-induced ectopic tails (arrow). (H) Ectopic tails (arrow) induced by Gli3 or Gli2 (not shown) lack floor plate (fp) or endodermal cells as judged by the absence of HNF-3β expression. The endogenous floor plate is labeled. In all panels, arrows show the position of the ectopic tails. Whole mounts show cleared embryos in F,G. Anterior is towards the left except in (H). All embryos are shown at tadpole stages (stages ~32-34).

included Xen1$^+$ (Ruiz i Altaba, 1992) neural tissue (Fig. 3G; *n*=10/13), but this was not of floor plate character judging by the lack of HNF-3β expression (Fig. 3H; *n*=0/5 and not shown), which is consistent with the lack of notochord. Because HNF-3β is also normally expressed in endoderm (Ruiz i Altaba et al., 1995), this marker also confirmed the lack of endodermal cells in the induced tails.

Gli2 repressors inhibit *brachyury* in the marginal zone

To test the involvement of Gli function in normal *brachyury* expression, we have used Gli repressor derivatives (Dai et al., 1999; Ruiz i Altaba, 1999a; Shin et al., 1999; Sasaki et al., 1999), and targeted them to the marginal zone, the site where the embryonic mesoderm forms and *Xbra* is endogenously expressed. Repressor forms of Gli2 and Gli3 lack C-terminal sequences, are similar in structure and function to the repressor form of the *Drosophila* Gli homolog cubitus interruptus (ci; Aza-Blanc et al., 1997; Ruiz i Altaba 1999a) and the Gli3 repressor used here has the same size as a putative endogenous repressor (Wang et al., 2000). Since the repressor forms of Gli2 (Gli2C'Δ) and Gli3 (Gli3C'ΔClaI) give similar results in our assays, we have used mainly Gli2C'Δ. As expected, control experiments in which Gli2C'Δ and Gli2 were co-expressed in the animal pole resulted in the inhibition of ectopic tail induction and in the development of normal tadpoles (*n*=10, not shown), further confirming the specificity of Gli repressors (see Ruiz i Altaba, 1999a; Liu et al., 1998).

Expression of Gli2C'Δ (Fig. 4A; stage 10+, *n*=35/35, Myc$^+$) or Gli3C'ΔCla1 (not shown, stage 10+, *n*=8/8, Myc$^+$) in the marginal zone resulted in the complete loss of endogenous *Xbra* mRNA in cells expressing Myc-tagged Gli proteins. Repression was also observed in embryos injected with as little as 50 pg of *Gli2C'*Δ RNA (*n*=7/29 repressions, Myc$^+$, not shown). In contrast, expression of a similar form of Gli1 (Gli1C'ΔPst1) did not affect *Xbra* expression (not shown, *n*=0/9 repressions, Myc$^+$). This result is in agreement with the lack of strong repressor activity in Gli1 (Ruiz i Altaba, 1999a; Sasaki et al., 1999; Dai et al., 1999) and serves as control for the loss of *Xbra* expression by truncated Gli2 and Gli3 proteins. Since these repressor forms have been shown to inhibit the function of any co-expressed activating Gli protein, our results suggest that Gli function is involved in endogenous *Xbra* expression. Embryos injected with Gli2C'Δ in the ventral marginal zone also showed loss of ventral mesodermal markers, such as *Msx1* (stage ~10.5, *n*=15/17, Myc$^+$, not shown), a result consistent with the requirement of brachyury for mesodermal identity (Smith et al., 1991; Cunliffe and Smith, 1992). To further confirm the specificity of the repressor action, we have tested the function of a chimeric Gli2 protein bearing the repressor domain of *Drosophila* engrailed linked only to the zinc-finger domain of Gli2 (Gli2EnR). Gli2EnR has been previously shown to inhibit activating Gli2 function (Brewster et al., 1998). Expression of Gli2EnR, like that of Gli2C'Δ, inhibited endogenous *brachyury* expression in the marginal zone (*n*=3/3, Myc$^+$; not shown).

Inhibition of endogenous *brachyury* expression by Gli repressors is not compensated by activation of TGFβ/Smad signaling

We tested whether activation of signaling pathways known to be involved in mesodermal development could counteract the inhibition of *brachyury* expression by Gli repressors. We first tested activation of the TGFβ pathway, as *Xbra* has been shown to be induced by TGFβ family signals such as activin (Latinkic et al., 1997) and Gli repressors have been shown to associate with Smads (Liu et al., 1998). Gli2C'Δ was therefore co-expressed in the marginal zone with either an activated type I activin receptor (Chang et al., 1997) or activated Smad2 fused to β-galactosidase (Baker and Harland 1996), both known to signal in a constitutive manner, and mimic activin and nodal type signals (Massagué, 1998). Control experiments showed

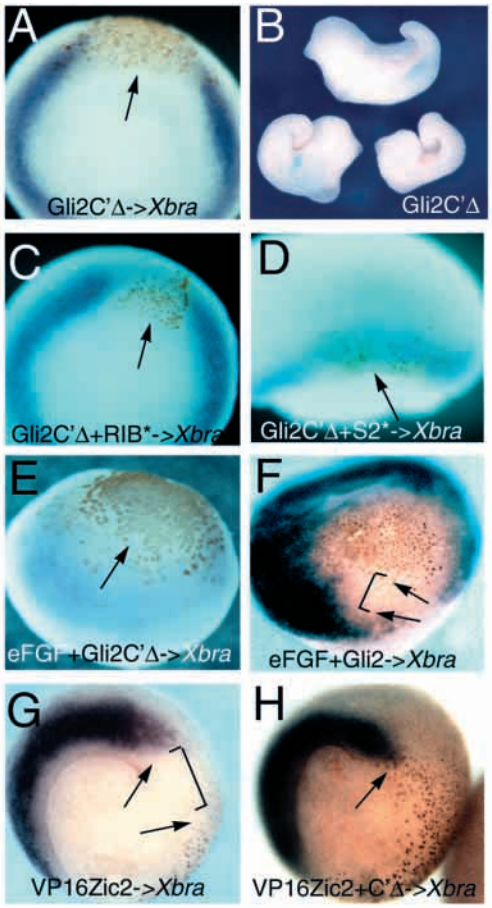

Fig. 4. Effects of repressor and activators on *Xbra* expression and involvement of Gli2 in the FGF-brachyury pathway. (A) Expression of Gli2C'Δ in the marginal zone of a stage ~10 embryo inhibits endogenous *Xbra* expression. Note that the arc of Myc label (brown) coincides with the gap (arrow) in *Xbra* expression (purple). (B) Posterior deficiencies in embryos (stage ~32) injected with *lacZ* plus *Gli2C'*Δ. (C) Inhibition of *Xbra* expression (arrow) by Gli2C'Δ in a stage ~10 embryo is not rescued by co-expression of activated activin type I receptor (RIB*). (D) Inhibition is also not rescued by co-expression of activated Smad2-lacZ fusion (S2) in a stage ~10 embryo. Note the light-blue β-gal product overlapping the Myc label in the *Xbra* gap (arrow). (E) Gli2C'Δ inhibits ectopic *Xbra* expression (arrow) induced by co-injected eFGF in a cell-autonomous manner in the animal cap of a stage ~10 embryo. Note the light-purple label around Myc-labeled (brown) cells in the animal cap. (F) Full-length Gli2 inhibits *Xbra* (arrows and bracket) by co-expressed eFGF in a stage ~11 embryo. Note the gap (bracket) between Myc-expressing and *Xbra*-expressing cells, indicating non-cell-autonomous repression. (G) Inhibition of endogenous *Xbra* expression at a distance from cells expressing the activator VP16Zic2 protein. The bracket shows the region of non-cell-autonomous repression of *Xbra* in between cells maintaining *Xbra* and cells inheriting the injected Myc-labeled material (arrows). (H) Gli2C'Δ (C'Δ) inhibits the non-cell-autonomous repression of *Xbra* by co-expressed VP16Zic2. The arrow indicates the junction of *Xbra* expression and Myc label, as in A. Embryos in (A,C,G,H) show vegetal views. (B) shows a lateral view with anterior towards the left (top and bottom right embryos) or towards the right (bottom left embryo). (D,F) show views of the marginal zone. (E) shows a view of the animal pole.

that the activated receptor or activated Smad2 could induce secondary axes and ectopic expression of *Xbra* (not shown). Neither the activated receptor (Fig. 4C; *n*=24/25 suppressions, Myc$^+$), nor the activated Smad2 proteins (Fig. 4D; *n*=18/19 suppressions, Myc$^+$) were able to rescue the loss of *Xbra* expression caused by co-expressed Gli repressors, and they did not induce secondary axes (not shown). These results suggest that injected Gli2 repressors act downstream of TGFβ/Smad signaling.

Gli2 functions in the FGF-brachyury pathway

The ability of Gli2 to induce ectopic tail formation in ectoderm is similar to that of FGF-induced posterior mesoderm (Ruiz i Altaba and Melton, 1989b). Moreover, FGF (eFGF) and brachyury have been shown to function in a regulatory loop required for mesodermal maintenance (Isaacs et al., 1994; Schulte-Merker and Smith, 1995; Griffin et al., 1995). Together, these findings raised the possibility that Gli2 could be acting downstream of FGF to maintain *Xbra* expression in mesoderm.

We first tested whether Gli function is required to mediate *Xbra* induction by eFGF in ectodermal cells. Co-expression of eFGF and Gli2C'Δ resulted in the induction of *Xbra* transcription in a halo around Myc-tagged Gli2C'Δ-expressing cells (Fig. 4E; stage 11, *n*=12/12, Myc$^+$). We think this is due to the cell-autonomous repression of *Xbra* by Gli2C'Δ and the non-cell-autonomous induction of *Xbra* by eFGF. Low levels of pre-existent *Glis* found in the animal cap (see below, Lee et al., 1997) could account for the initial induction of *Xbra* by eFGF. Subsequently, all ventral mesodermal genes, including *Gli2*, are expected to be turned on in the induced mesoderm (e.g. Isaacs et al., 1994). These results suggest that FGF signaling requires positive Gli function to induce *Xbra* expression in ectodermal cells. The action of Gli2 in the FGF-brachyury pathway implies that *Gli2* should be inducible by FGF and brachyury. Indeed, expression of injected Xbra in ectoderm, which activates eFGF (Pownall et al., 1996), resulted in the ectopic expression of *Gli2* (Fig. 2E; stage 12-13, *n*=9/19 ectopic) and other mesodermal markers such as *SalB* (Fig. 2F; stage ~12-14, *n*=13/22). Similarly, expression of eFGF in animal caps resulted in *Gli2* transcription (Fig. 5D), although this was sensitive to CHX, indicating that the initial activation of *Gli2* by FGF is not direct (data not shown).

The ability of Gli repressors to inhibit *Xbra* expression, together with the requirement of Xbra for mesodermal development (Cunliffe and Smith, 1992), suggests that the affected embryos should gastrulate abnormally and mimic the phenotype of embryos compromised for Xbra function. To investigate this possibility, embryos were injected into the equatorial region of one or two cells at the two- to four-cell stage with mRNAs encoding the Gli repressors alone or along with *lacZ* mRNA as a tracer. Injection of Gli2C'Δ in the equator of two cells at the two-cell stage resulted in gastrulation defects and embryos lacking normal posterior structures (Fig. 4B; *n*=19/26 embryos). Embryos that received the injected material in prospective ectoderm developed normally (not shown). Embryos that had β-gal expression only in the trunk mesoderm often showed slightly deformed somites (*n*=5/7, not shown), consistent with a later role of Gli2 in somitogenesis (e.g. Borycki et al., 1998; Karlstrom et al.,

1999). Inhibition of Gli2 function therefore appears to alter normal ventroposterior development.

Gli2 transiently inhibits *brachyury* at early stages

Requirement of Gli2 in normal mesodermal development is supported by the inhibition of *Xbra* by Gli2 repressors. Surprisingly, expression of full-length Gli2 alone in the marginal zone at stage 10 also repressed *Xbra* (not shown; *n*=15/15, Myc$^+$). To test whether repression of *Xbra* by full-length Gli2 at early stages was due to activator or repressor functions, we used a chimeric form of Zic2, a Gli-related protein, bearing the VP16 transactivation domain fused to the Zic2 Gli-type zinc-finger domain. This protein, VP16Zic2, is a strong transcriptional transactivator and has previously been shown to act exactly as activating full-length Gli2 in multiple assays (Brewster et al., 1998). Injection of VP16Zic2 also resulted in the repression of endogenous *Xbra* (Fig. 4G; *n*=60/60), indicating that both the activator and repressor functions inhibit early *Xbra*. However, repressor and activator proteins appear to repress *Xbra* via different mechanisms. Repression of endogenous *Xbra* by Gli2C'Δ was always (*n*=48/48) cell-autonomous (Fig. 4A), whereas repression by Gli2 (*n*=15/15) or VP16Zic2 (*n*=60/60) was always non-cell-autonomous with a range of several (>10) cell-diameters (Fig. 4G and not shown). Similarly, Gli2 repressed the ectopic expression of *Xbra* by co-injected eFGF in a non-cell-autonomous manner (Fig. 4F; *n*=54/54, Myc$^+$). Gli2 activators may thus induce both *Xbra* and a strong repressor of *Xbra* at early stages. At later stages, this repressor may be inactive, allowing Gli2 to induce *Xbra*. At both times, Gli2C'Δ would be expected to inhibit *Xbra*. This model predicted that co-injection of VP16Zic2 and Gli2C'Δ should diminish non-cell-autonomous repression as Gli2C'Δ might directly repress the postulated secreted inhibitor. Indeed, co-injection of VP16Zic2 and Gli2C'Δ at equal ratios showed a decrease in the number of embryos with non-cell-autonomous *Xbra* repression (Fig. 4H; *n*=51/80, Myc$^+$). The persistence of some non-cell-autonomous repression may be due to the very high potency of the VP16 transactivation domain.

brachyury and *Xhox3* are targets of Gli2

To corroborate the results obtained in whole embryos, we tested whether Gli2 could induce *Xbra* expression in explanted animal caps. In stage ~14 animal caps, Gli2 induced the expression of *Xbra* even in the presence of cycloheximide (CHX, Fig. 5A), a protein synthesis inhibitor extensively used in this type of assay to test for direct activation of target genes. This result shows that *brachyury* is directly regulated by Gli2. We note also that low levels of *Gli2* are present in the untreated

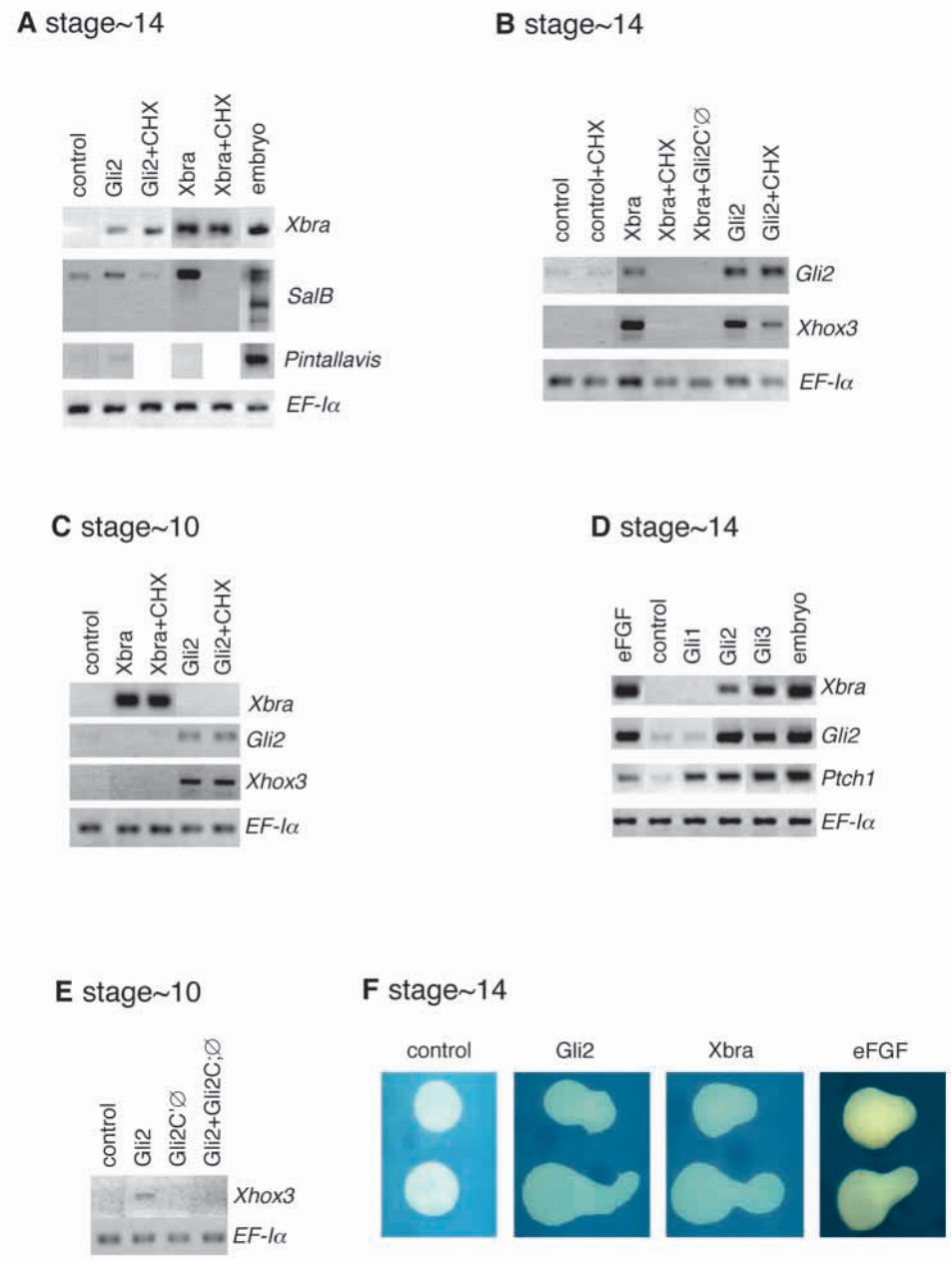

Fig. 5. Gli2 and brachyury induce mesoderm formation and elongation in isolated animal caps. (A-E) RT-PCR analyses of animal cap explants injected and treated in various ways. The stage is given and the injected RNAs or treatments are shown above each column. The name of the tested genes is given to the left of each row at the corresponding position. Note that *SalB* shows three specific products in whole embryos but only one in animal caps. **(**F) Morphological appearance of uninjected, *Gli2*-, *Xbra*- and *eFGF*-injected albino animal caps cultured to the equivalent of stage 14. Note the pronounced elongation of caps injected with *Gli2* or *Xbra*. Two representative caps are shown in each case.

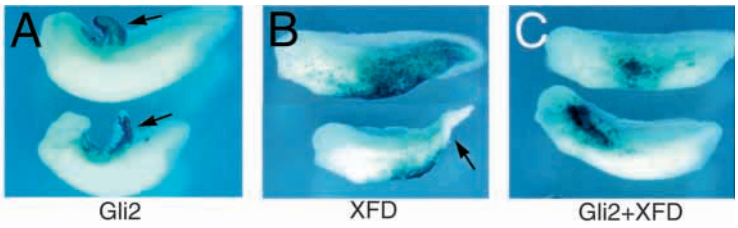

Fig. 6. Gli2 function requires FGF signaling. (A) Induction of ectopic tails (arrows) in tadpoles after injection of *Gli2* plus *lacZ*. (B) Expression of XFD in posterior mesoderm antagonized proper posterior development (arrow; Amaya et al., 1991). (C) XFD inhibits ectopic tail induction by co-expressed Gli2. Each panel shows lateral images of two tadpoles at stages ~32-34 with similar phenotypes. Anterior is towards the left.

caps (Fig. 5B-D). The early inhibition of *Xbra* seen in response to Gli2 in whole embryos (see above) raised the possibility that a similar change in competence could be detected in animal caps. Gli2-injected and control animal caps were then harvested at early (stage ~10-10.5) and late (stage ~13-14) gastrula stages from the same injected batch and tested for *Xbra* expression. At early stages, Gli2 did not induce *Xbra* **(**Fig. 5C). One possibility, as explained above, is that, Gli2 may be activating not only *Xbra* but a potent *Xbra* repressor. However, because *Xbra* is not induced by Gli2 in the presence of CHX at stage 10 (Fig. 5C), which would prevent the synthesis of the putative repressor, this result suggests that repression of *Xbra* includes changes in pre-existing repressors, and/or that activation of *Xbra* involves Gli2 modifications.

Brachyury expression in stage ~14 animal caps (Fig. 5A) is a reliable indication of mesodermal development (Cunliffe and Smith, 1992). Another indication is the elongation of caps containing mesoderm (Symes and Smith, 1987). Consistent with their ability to induce mesoderm, elongation of animal caps was observed after injection of eFGF (*n*=8/11), Xbra (*n*=13/15) or Gli2 (*n*=7/8), whereas uninjected control caps remained round (*n*=0/13; Fig. 5F). Gli3, but not Gli1, also induced *Xbra* and *Gli2* (Fig. 5D), consistent with the expression of *Gli2* and *Gli3*, but not *Gli1*, in ventroposterior mesoderm and the ability of the first two to induce ectopic tail formation.

As control for Gli activity in our animal cap assay, we sought to test for the induction of *patched* (*Ptch*) genes. Degenerate RT-PCR analyses (Takabatake et a., l997) yielded an 882bp-long frog *Ptch1* clone (GenBank Accession number AF254386) showing 71% identity to human *PTCH1*, 73% identity to chick *PTCH1*, 71% identity to mouse *Ptch1* and 68% identity to newt *ptch1*. In contrast, it only showed 48% identity to mouse *Ptch2*. Using this clone, we demonstrated that all three Gli proteins were able to induce *Ptch1* expression in animal caps (Fig. 5D), indicating a shared activity and showing that the inability of Gli1 to induce *Xbra* is not due to its lack of function.

The ability of Gli2 to induce ectopic tails and *Xbra* expression raised the possibility that it could regulate the function of genes previously implicated in ventroposterior development. Indeed, we found that Gli2 induced *Xhox3*, a homeobox gene required for posterior specification (Ruiz i Altaba and Melton, 1989a,b) both at stage ~10 and at stage ~14 (Fig. 5B,C). Induction at stage ~10 indicates that the Gli proteins are active at this time. *Xhox3* activation by Gli2 was not affected by CHX treatment and it therefore further supports a role of Gli2 in directly controlling ventroposterior fates as Xhox3 is a critical factor in ventroposterior development (Ruiz i Altaba et al., 1991). Consistent with this finding, Gli2C'Δ was able to inhibit the induction of *Xhox3* at stage ~10 by co-injected Gli2 **(**Fig. 5E**)**. Brachyury also induced *Xhox3* at late stages (Fig. 5B), but this was indirect and appeared to depend on Gli2, since CHX treatment or co-expression with Gli2C'Δ inhibited its expression (Fig. 5B). In addition, the induction of *Gli2* by brachyury is blocked by co-expressed Gli2C'Δ, providing support for the possibility of *Gli2* autoregulation or for the requirement of concurrent function of the Gli2-brachyury-FGF loop for *Gli2* expression (Fig. 5B).

We have also analyzed the expression of *SalB* by RT-PCR as an additional mesodermal marker. In whole embryos, several specific bands were detected (see also Hollemann et al., 1996). Whereas the lower band in Fig. 5A is predicted to represent the *SalB* transcript (Fig. 1B), the more prominent upper band represents a putative full-length, non-differentially spliced mRNA, predicted to exist based on analyses of *Xsal1* (Hollemann et al., 1996). This was the only species detected in animal caps, and was only induced indirectly by Gli2 and Xbra (Fig. 5A). Thus, the *SalB* transcript originally identified in our cDNA screen may be a rare form originating from a gene exhibiting complex transcriptional regulation. Since Xbra induced *SalB* at higher levels than did Gli2 (Fig. 5A), it is possible that Gli2 regulates *SalB* through Xbra.

As a control for the character of the induced mesoderm in animal cap assays, we used the expression of *pintallavis*, a winged-helix gene specifically expressed dorsally in the organizer (Ruiz i Altaba and Jessell, 1992; Dirksen and Jamrich, 1992). Whereas *pintallavis* expression was detected in whole embryos, it was not induced by Gli2, by Xbra or detected in control animal caps (Fig. 5A), a result consistent with the involvement of Gli2 in ventroposterior development. As a control for mRNA abundance, the expression of the ubiquitous gene *EF-Iα* (Krieg et al., 1989) was always tested (Fig. 5A-D).

Posterior induction by Gli2 requires concurrent FGF activity

A possible simple model of the regulatory network involving FGF, brachyury and Gli2 could suggest that Gli2 acts as the effector of the FGF-brachyury loop in posterior development. In this case, injected Gli2 would bypass FGF-brachyury signaling. To test this idea, we co-expressed Gli2 and XFD, a dominant-negative FGF receptor, and assayed for ectopic tail formation. Injection of *XFD* and *lacZ* resulted in posterior deficiencies when the injected material was localized posteriorly (Fig. 6B; *n*=41/73; Amaya et al., 1991) and Gli2 alone induced ectopic tail-like structures (Fig. 6A). Co-injection of *XFD* plus *Gli2* and *lacZ* prevented the formation of ectopic tails (Fig. 6C, *n*=0/69). Similarly, XFD also prevented ectopic tail formation by Gli3 (not shown, *n*=0/48). These results thus indicate the importance of concurrent FGF signaling for posterior development induced by Gli proteins.

DISCUSSION

The findings presented here indicate that Gli2 participates in the elaboration of the posterior fates by mediating FGF signals during A-P patterning. We show that Gli proteins are important components of a network for ventroposterior mesodermal maintenance: they act in the FGF-brachyury regulatory loop by regulating *brachyury* and responding to FGF. Gli2 induces ventroposterior fates by directly controlling *Xhox3* (*Evx1*), a homeobox gene necessary and sufficient for posterior and tail development. However, the final induction of posterior development still requires concurrent FGF signaling, suggesting the nonlinearity of this network. More generally, our results show that Gli2 and Gli3, proteins involved in HH signaling, also act in a different signaling pathway, suggesting an integration of FGF and HH signaling events at the level of Gli protein function.

Gli2 and the FGF-brachyury pathway

Interference and gain-of-function analyses indicate that Gli2 (and later Gli3) functions in the FGF-brachyury pathway downstream of its initial induction by TGFβ family signals (Amaya et al., 1993; Isaacs et al., 1994; LaBonne and Whitman, 1994; Cornell et al., 1995; Schulte-Merker and Smith, 1995; Rodaway et al., 1999). Indeed, inhibition of endogenous FGF, Gli2 or brachyury results in similar posterior deficiencies (this paper; Amaya et al., 1991; Kroll and Amaya, 1996; Conlon et al., 1996). Moreover, misexpression of Gli2 (this paper), Xbra (Tada et al., 1997), eFGF (Pownall et al., 1996) or laloo, a Src-family kinase that is involved in the intracellular transduction of the FGF signal (Weinstein et al., 1998), results in nearly identical induction of ectopic tails. The more prominent defects in tail versus trunk development in embryos injected with Gli2 repressors is also consistent with the involvement of Xbra in tail rather than trunk formation (e.g. Griffin et al., 1995, 1998).

Gli3, but not Gli1, seems to have a similar function to Gli2, although it is expressed at later stages also in ventroposterior mesoderm, suggesting that it co-operates with Gli2 at these later times. Since Gli3 also induces *Gli2*, some of its functions could be mediated through the latter. Gli proteins appear to be able to yield cleaved repressor forms (Dai et al., 1999; Ruiz i Altaba, 1999a; Shin et al., 1999; Wang et al., 2000). However, little is known about their endogenous regulation, and functional properties are deduced from experiments using full-length and C-terminally truncated forms (that may resemble the endogenous repressors), and through their homology with the repressor form of CI in flies (Aza-Blanc et al., 1997). In this context, the studies presented here indicate that Gli2 and Gli3 activators are functional in the early-ventral/later-posterior mesoderm. A role of any possible endogenous repressor, which could involve repressing dorsoanterior fates, remains to be determined.

Differential response to Gli2 in early embryos

Gli2 or activating forms that mimic its function induce *brachyury*. At early gastrula stages, however, they inhibit *Xbra* expression. This repression appears to result from the induction of an unidentified non-cell-autonomous inhibitor. The function of this change in *Xbra* regulation is not known, but it may parallel normal development since the early endogenous expression of *Xbra* (and the related *Xbra3* gene) is itself graded along the D-V axis being delayed ventrally (e.g. Smith et al., 1991; Ruiz i Altaba and Jessell, 1992; Hayata et al., 1999). It is conceivable that a difference in the timing of *Xbra* expression contributes to the elaboration of ventral character. Surprisingly, *Xhox3*, but not *Xbra*, is readily induced at early stages by Gli2. Because the domains of expression of *Xhox3* and *Xbra* overlap at early stages (Ruiz i Altaba et al., 1991; Smith et al., 1991), and Xbra is required for mesoderm formation (Cunliffe and Smith, 1992), the earliest expression of *Xhox3* induced by Gli2 may be ineffective. Alternatively, it may predispose the mesoderm to a posterior fate.

Gli regulation and integration of signaling inputs

Previous work has shown that *Gli2* can be induced by SHH signaling in frog embryos, and that it can mediate some of the effects of SHH (Ruiz i Altaba, 1998). We show here that FGF also induces *Gli2*, although it remains unclear which factors directly initiate its expression in mesoderm, as this is difficult to separate from the general induction of mesoderm by FGF or TGFβ family signals.

While Gli2/3 function in mesoderm may have nothing to do with HH signaling, *Hh* genes have been reported to be expressed at low levels throughout the gastrula marginal zone (Ekker et al., 1995), raising the possibility that Gli2 and Gli3 activity in mesoderm could be responsive to HH signals by analogy with some of its later roles in neural development. For example, a low tonic HH signal throughout the marginal zone could attenuate the formation of putative Gli3 repressors, a process regulated by the SHH signaling pathway (Dai et al., 1999; Ruiz i Altaba, 1999a; Wang et al., 2000), thus allowing Gli2 and Gli3 activator forms to function in ventroposterior development. The fact that misexpression of HHs at early stages has no obvious consequence on mesodermal development (Ekker et al., 1995; Ruiz i Altaba et al., 1995; Sampath et al., 1997) could be consistent with this possibility if repressor forms were not required in mesoderm. In mice, loss of SHH, Gli2 or Gli3 function does not appear to affect the early embryonic mesoderm (Chiang et al., 1996; Hui and Joyner, 1993; Mo et al., 1998; Ding et al., 1999; Matise et al., 1999; Park et al., 2000), possibly indicating that Gli proteins could have partially divergent roles in different organisms.

The role of Gli2 in FGF signaling, the ability of FGF and SHH to induce its expression and its partial mediation of SHH functions (Ruiz i Altaba, 1998; Sasaki et al., 1999) suggest a mechanism for a possible integration of FGF and HH signaling in tissues in which these signals act on the same Gli-expressing cells. SHH can act through Gli1, and Gli3 has an antagonistic relationship with SHH/Gli1. In contrast, SHH can also act through Gli2 in some contexts, but in others, Gli2 can instead antagonize the actions of SHH and Gli1 (reviewed in Ruiz i Altaba, 1999b). A context-dependent function of Gli2 could therefore underlie the sometimes synergistic and sometimes antagonistic effects of FGFs and HHs. Similarly, antagonism between HH and FGF signaling could result from their use of Gli1 and Gli3, respectively. Our model may be particularly relevant for Gli-expressing precursor cells. For example, SHH is a known mitogen for cerebellar granule precursors and FGF can partially inhibit this effect (Dahmane and Ruiz i Altaba, 1999; Wallace, 1999; Weschler-Reya and Scott, 1999). Because Wnt signaling has been recently suggested to affect

Gli2 and *Gli3* expression in chick somites (Borycki et al., 2000), a challenge of ongoing studies is to elucidate how different signaling inputs regulate Gli function in vertebrate development and disease.

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