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Wnt signals are targets and mediators of Gli function

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There is growing evidence that Gli proteins participate in the mediation of Hedgehog and FGF signaling in neural and mesodermal development. However, little is known about which genes act downstream of Gli proteins. Here we show the regulation of members of the *Wnt* family by Gli proteins in different contexts. Our findings indicate that Gli2 regulates *Wnt8* expression in the ventral marginal zone of the early frog embryo: activating Gli2 constructs induce ectopic *Wnt8* expression in animal cap explants, whereas repressor forms inhibit its endogenous expression in the marginal zone. Using truncated Frizzled and dominant-negative Wnt constructs, we then show the requirement of at least two Wnt proteins, Wnt8 and Wnt11, for Gli2/3-induced posterior mesodermal development. Blocking Wnt signals, however, inhibits Gli2/3-induced morphogenesis, but not mesodermal specification. Gli2/3 may therefore normally coordinate the action of these two Wnt proteins, which regulate distinct downstream pathways. In addition, the finding that Gli1 consistently induces a distinct set of *Wnt* genes in animal cap explants and in skin tumors suggests that Wnt regulation by Gli proteins is general. Such a mechanism may link signals that induce Gli activity, such as FGFs and Hedgehogs, with Wnt function.

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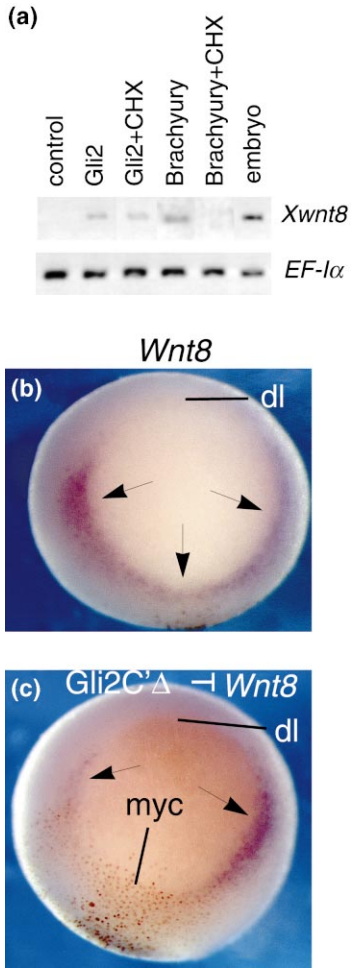
Results and discussion

Gli zinc finger transcription factors are targets and mediators of Hedgehog signaling [1–3] (see [4] for a review). In addition, Gli2 and Gli3, but not Gli1, also act downstream of FGF signaling in the early ventro-posterior (VP) embryonic mesoderm of the developing frog embryo, where they regulate *Brachyury* and *Xhox3* [5]. Brachyury is a T-box transcription factor required for mesodermal development [6, 7], and Xhox3 (Evx1) is a homeodomain protein required for posterior identity [8, 9]. Here, we test the hypothesis that *Wnt* genes are targets and mediators of Gli function since Wnt signaling has been implicated in ventral and posterior development (e.g., [10–18]) and Wnt function may cooperate with Xhox3 [19]. In addition, there is a general correlation in the expression of *Gli* and *Wnt* genes [1, 20], and similar posterior-deficient phenotypes are observed after inhibition of endogenous FGF, Gli2/3, and Wnt function [5, 11, 15, 16, 21–23]. Since the expression of *Wnt8* in the ventral marginal zone of the early gastrula [13, 14, 21, 22] is similar to that of *Gli2* [5], we have focused first on this *Wnt* gene.

To test whether Gli proteins regulate *Wnt8* gene expression, we used animal caps (undifferentiated ectodermal explants) from injected frog embryos. Expression of injected Gli2 or Gli3 in animal cap cells resulted in the induction of *Wnt8*. This induction appears to be direct, as it occurred even in the presence of cycloheximide (CHX), a widely used, potent protein synthesis inhibitor (Figure 1a; data not shown). As a control, *Wnt8* induction by Brachyury was sensitive to CHX (Figure 1a) and, thus, was indirect. To test whether Gli2 function is required for the endogenous expression of *Wnt8*, we used a C-terminally truncated Gli2 repressor (Gli2C′Δ) that acts in a dominant-negative manner [24]. Expression of Gli2C′Δ in the ventral marginal zone inhibited endogenous *Wnt8* (Figures 1b,c; n = 20/20 repressions), suggesting that Gli2 plays a critical role in *Wnt8* regulation. We also tested whether ectopic Wnt8 was able to induce *Gli* gene expression in animal cap cells but found no effect (data not shown), indicating the absence of a regulatory feedback loop.

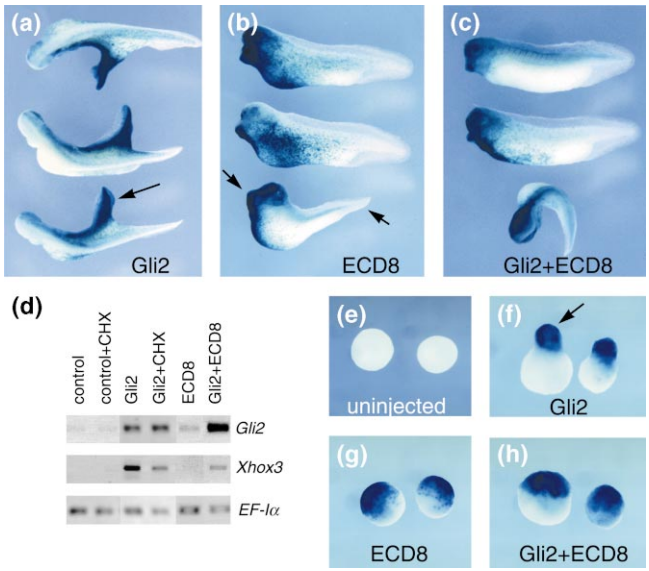
The regulation of *Wnt8* by Gli2, together with their involvement in mesodermal development [5, 13, 14, 21, 22], suggested the possible participation of Wnt8 in the specification and/or morphogenesis of Gli2-induced VP mesoderm. To test this idea, we expressed the extracellular domain of the Wnt receptor Frizzled 8 (ECD8), which is a potent general Wnt antagonist [16], together with Gli2. Expression of Gli2 led to the development of ectopic tail-like structures, hereafter referred to as tails [5] (Figure 2a, n = 12/48). Expression of ECD8 and β-gal (used in all cases as a lineage tracer) caused the expected posterior defects and the concomitant enlargement of anterior structures [16] (Figure 2b, n = 63/66). In contrast, coexpression of Gli2 plus ECD8 did not result in ectopic tail development (Figure 2c, n = 0/109). ECD8 also impaired

Figure 1



Gli2 regulates *Wnt8*. **(a)** RT-PCR analyses of *Wnt8* expression in isolated animal caps (stage ~14) injected with *Gli2* or *Brachyury* RNAs or uninjected and treated with or without cycloheximide (CHX), as labeled on top. Expression of *EF-1α* was measured as a control for RNA recovery. **(b)** Normal expression of *Wnt8* in the ventral and lateral marginal zones (indicated by an arrow) of a stage ~10.5 embryo. The dorsal lip (dl) is denoted. Note the continuous expression in the ventral and lateral areas (purple). **(c)** Expression of injected Gli2C′Δ in the ventral marginal zone inhibits the endogenous expression of *Wnt8*. Note the anti-myc labeling (brown) in cells expressing myc-tagged Gli2C′Δ protein. Arrows demarcate the areas of normal expression (purple) in areas not exhibiting myc labeling. Embryos in (b,c) show vegeto-lateral views.

Figure 2



Wnt signaling is required for Gli2-induced morphogenesis, but not VP mesodermal specification. **(a)** Induction of ectopic tail-like structures (indicated by an arrow) in tadpoles after injection of *Gli2* plus *LacZ*. Note the localization of β-gal reactivity in the supernumerary tail. **(b)** Injection of the truncated Frizzled 8 receptor ECD8 antagonizes posterior development and promotes anterior development [16] (indicated by arrows). **(c)** ECD8 inhibits tail induction by Gli2. (a–c) show lateral images of tadpoles at stages ~32–34. The anterior is to the left. **(d)** RT-PCR analyses of isolated animal caps (stage ~14) injected or treated as described on top, testing for the expression of *Gli2*, *Xhox3*, or *EF-1α*. Note that while there appears to be a little less *Xhox3* RNA in the Gli2 + ECD8 sample, it is still expressed, indicating that ECD8 does not prevent its activation and, thus, posterior specification by Gli2. Negative controls are untreated sibling animal caps. **(e–h)** Animal caps at stage ~14 (e) left untreated, (f) injected with *Gli2* and *LacZ*, (g) *ECD8* and *LacZ*, or (h) *Gli2* and *ECD8* plus *LacZ*. The images show the caps after β-gal staining.

the tail-inducing function of Gli3 (data not shown; n = 30/66 for Gli3 alone, and n =14/77 for Gli3 plus ECD8). These results show that inhibition of Wnt signaling blocks ectopic tail induction in vivo by Gli2/3, suggesting that Wnt function is required for Gli2/3-induced VP mesodermal development.

In animal cap cells, however, ECD8 did not alter the ability of coexpressed Gli2 to induce expression of *Xhox3* (Figure 2d), a critical VP specification gene [8, 9]. As a control, ECD8 alone did not induce the expression of *Xhox3* or *Gli2* (Figure 2d). Gli2 is also able to induce neurogenesis in competent ectoderm [1, 5]. In this context, ECD8 was also unable to inhibit neuronal induction by Gli2 (n = 4/7 positive for *N-tubulin* with Gli2 alone, and n = 13/15 positive for *N-tubulin* with Gli2 plus ECD8; data not shown), further suggesting that Wnt function may not be required for Gli2-induced cell specification.

Untreated animal caps become spherical after culture (Figure 2e). In contrast, mesoderm induction in sibling animal caps by injected Gli2 results in a morphogenetic program that drives their elongation [5] (Figure 2f, n = 7/8). Interestingly, caps injected with ECD8 alone (used as controls, Figure 2g) and animal caps coexpressing Gli2 and ECD8 failed to elongate (Figure 2h, n = 0/8; and data not shown). The specificity of this effect is shown by the ability of Wnt8 to rescue the morphogenesis induced by Gli2 in the presence of ECD8 (87% elongation

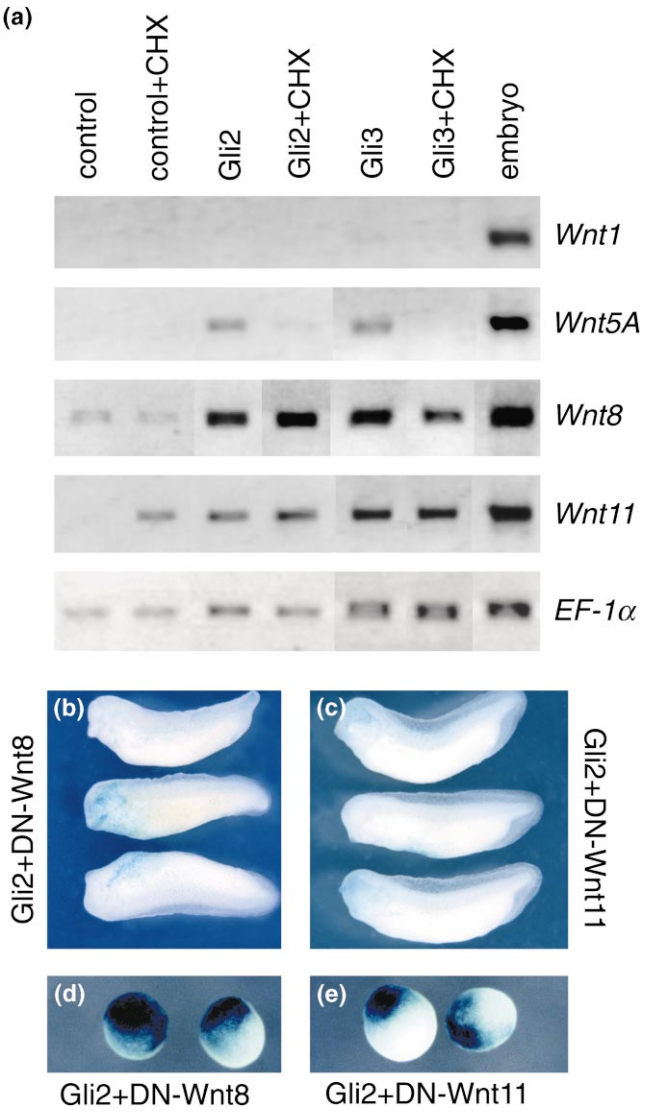
of Gli2-injected caps, n = 15; 19% elongation of Gli2 + ECD8-injected caps, n = 16; 71% elongation of Gli2 + ECD8 + Wnt8-injected caps, n = 14; data not shown). These findings indicate that Wnt activity is required for Gli2-induced VP morphogenesis.

In addition to *Wnt8*, other *Wnt* genes could also participate in the mediation of posterior morphogenesis induced by Gli2/3. Analyses of the expression of a variety of *Wnt* genes in Gli2/3-injected animal caps showed that *Wnt1*, *2*, *3*, *3A*, *4*, *6*, *7A*, and *10* are not induced by Gli2/3, whereas *Wnt5A*, *7B*, *7C*, and *8B* are induced, albeit indirectly, since CHX prevents their upregulation (Figure 3a; and data not shown). In contrast, *Wnt11* [17], like *Wnt8*, is induced by Gli2/3 (Figure 3a). This induction may be direct, although the superinduction of *Wnt11* by CHX in uninjected caps (Figure 3a; [25]) makes direct regulation difficult to ascertain. *Wnt11* has been shown to be required for convergent extension movements during gastrulation [11, 15], being expressed in the circumblastoporal region that gives rise to posterior mesoderm [17]. A link between Wnt8 and Wnt11 with Gli2 is also suggested by the phenotypes of interference with the endogenous function of Wnt11 [11, 12, 15] or Wnt8 [21, 16], which show posterior deficiencies similar to those obtained after interference with endogenous Gli2 function [5].

To test the requirement of Wnt11 and Wnt8 for the morphogenesis of VP mesoderm induced by Gli2, we used dominant-negative (DN) forms that appear to be specific [12, 15, 21]. Coexpression of Gli2 and DN-Wnt8 in animal caps resulted in the inhibition of ectopic tail development in vivo (Figure 3b, n = 0/50 ectopic tails) and of animal cap elongation (Figure 3d, n = 3/17 elongated caps), as compared with those induced by Gli2 alone (data not shown, but see Figure 2a, n = 41/65 ectopic tails, and n = 8/9 elongation in animal caps). Coexpression of Gli2 and DN-Wnt11 yielded the same result (Figure 3c, n = 3/24 ectopic tails; Figure 3e, n = 4/20 elongated caps). As in the case of ECD8, the effect of DN-Wnts was rescued by Wnt coexpression (0% elongation of Gli2 + DN-Wnt8-injected caps, n = 15; 47% elongation of Gli2 + DN-Wnt8 + Wnt8-injected caps, n = 15; data not shown). Moreover, DN-Wnts or ECD8 did not inhibit morphogenesis nonspecifically, as they did not inhibit the dorsal type elongation induced by Activin signaling (48% elongation of animal caps expressing a type I activated Activin receptor (ALK4 T206E), n = 33; 69% elongation of ALK4 T206E + ECD8-injected caps, n = 39; 50% elongation of ALK4 T206E + DN-Wnt8-injected caps, n = 30; data not shown). VP morphogenesis triggered by Gli2/3 therefore specifically requires the action of the *Wnt8* and *Wnt11* genes. Other *Wnts*, such as *Wnt5A* [10], could also be involved at later stages.

The induction of vertebrate *Wnt* genes by Gli2/3 is reminiscent of the induction of *Wg* by Ci in *Drosophila* (reviewed in [26]), indicating that regulation of *Wnt* gene expression by Gli/Ci proteins is a common and ancient mechanism used in different contexts. To test whether such a mechanism extends to Gli1, a factor that responds

Figure 3



Wnt gene induction by Gli2 and Gli3, and the requirement of Wnt8 and Wnt11 for Gli2/3-induced VP morphogenesis. **(a)** Analyses of *Wnt* gene expression in isolated animal caps left untreated (control) or injected with Gli2 or Gli3 and treated or untreated with cycloheximide (CHX). Even though CHX has a slight superinduction effect on *Wnt11* in control animal caps, the high levels seen in Gli2- or Gli3-injected caps is maintained. **(b,c)** Coexpression of (b) *DN-Wnt8* or (c) *DN-Wnt11* with *Gli2* inhibits the tail-inducing activity of Gli2. Compare the lack of tails in these embryos with those shown in Figure 2a induced by expression of Gli2 alone. Images show lateral views of tadpoles at stages ~32–34. The anterior is to the left. **(d,e)** Animal caps at stage ~14 injected with (d) *Gli2*, *DN-Wnt8* plus *lacZ* or (e) *Gli2*, *DN-Wnt11* plus *lacZ*. Compare these to untreated controls and Gli2-injected caps (Figures 2e,f).

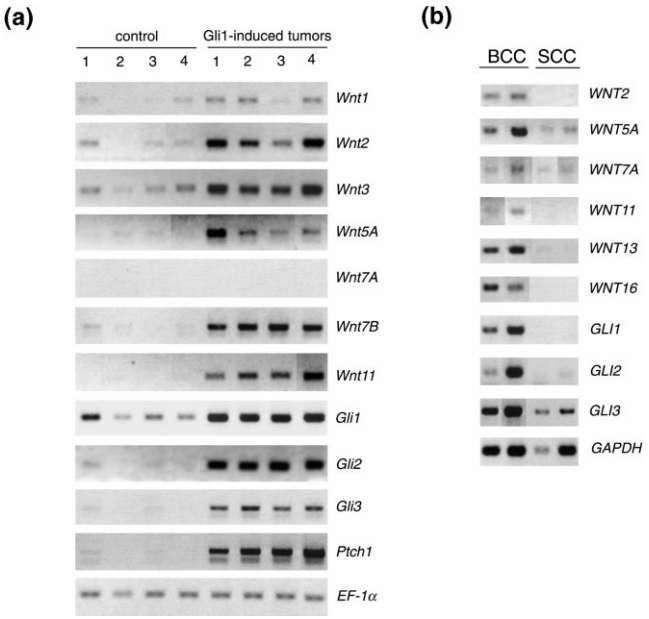
to and mediates HH signaling [1, 2, 4], but which does not appear to participate in FGF signaling in the early mesoderm [5], we used two different assays that test for the expression of *Wnt* genes. Expression of Gli1 in animal caps induced *Ptch1* expression [5] as well as the expression or the upregulation of *Wnt5A*, *7B*, *7C*, and *8* (but only that of *Wnt8* directly), but not that of *Wnt1*, *2*, *3*, *3A*, *4*, *6*, *7A*, *8B*, *10*, or *11* (data not shown), indicating that like Gli2/3, Gli1 induces a distinct set of *Wnt* genes.

We have extended these findings by asking whether Gli proteins can also lead to *Wnt* gene expression in a different context. Expression of human *GLI1* in epidermal cells leads to the endogenous activation of frog *Gli1* and the formation of basal cell carcinoma (BCC)-like skin tumors in late tailbud tadpole stages [27]. Such tumors were dissected from affected young tadpoles (stage ~30), tested for *Wnt* gene expression, and compared to the expression seen in contralateral normal epidermal samples. GLI1-induced tumors showed specific upregulation of *Wnt2*, *3*, *5A*, *7B*, and *11* as well as *Gli2*, *Gli3*, and *Ptch1* genes (Figure 4a). *Wnt1*, *3A*, *4*, *6*, *7A*, *7C*, *8*, *8B*, and *10* were not expressed or upregulated (Figure 4a; data not shown) in such tumors, but were expressed in total embryo samples used as controls (data not shown). The level of cDNA in each sample was determined by the expression of the housekeeping gene *EF-1α* (Figure 4a).

The relevance of our results with frog tumors is suggested by the findings that human sporadic BCCs, which derive from activation of the SHH-GLI pathway [27, 28], express a similar set of *Wnt* genes (Figure 4b). BCCs consistently expressed *Wnt2*, *5A*, *11*, *13*, and *16*, although only *Wnt2*, *11*, and *16* were not expressed in unrelated human skin squamous cell carcinomas (SCCs, which do not express *GLI1*) used as controls (Figure 4b; [27]). *Wnt6*, *7A*, and *14* were not consistently expressed in BCCs, but were expressed in human fetal brain samples used as a control (data not shown). All cDNA samples were controlled for RNA levels by the expression of the housekeeping gene *GAPDH*. Tumor content was measured by the expression of the general tumor marker *DDR1* (Figure 4b; data not shown; [29]). These findings thus indicate that Gli1 function leads to the expression of *Wnt* genes in different contexts.

While the role of the vertebrate *Wnt* family is beginning to be understood, much remains to be elucidated about their regulation. Here, we show that Gli proteins regulate the expression of different *Wnt* genes. During VP morphogenesis Gli2/3 directly activates *Wnt8* and possibly *Wnt11*, which in turn will stimulate the β-catenin and the Ca^{2+}/CamKII pathways, respectively (e.g., [12] and references therein, [15]). Since no single Wnt protein appears to be sufficient to induce tail formation efficiently (e.g., [10, 14, 19]), Gli2/3 could thus coordinate these two pathways to promote VP (tail) development. We show also that Gli1

Figure 4



Wnt gene induction by Gli1. **(a)** Analyses of *Wnt* gene expression in frog BCC-like epidermal tumors induced by expression of human GLI1. The results with four independent tumors and four unrelated and independent normal skin controls from stage ~30 embryos (when tumors can be cleanly dissected out) are shown. Additional samples (n = 4) showed the same results (data not shown). Note the predicted induction of *Gli* and *Ptch1* genes [27]. *EF-1α* was used as a control for total RNA recovery and levels. **(b)** Analyses of *Wnt* gene expression in independent human sporadic basal cell carcinoma (BCC) and human sporadic squamous cell carcinoma (SCC) tumor samples. BCCs express *GLI* and *PTCH1* genes [27] and SCCs are used here as a control tumor type unrelated to SHH/GLI signaling. The housekeeping gene *GAPDH* is used as a control for RNA levels. Analyses of 11 independent sporadic BCCs showed that *GLI1*, *GLI2, Wnt5A,* and *Wnt11* are expressed in all of them; *GLI3* is expressed in 10/11 cases; *Wnt7A* is expressed in *3/11* cases, and *Wnt13* and *Wnt16* are expressed in 10/11 cases (data not shown).

leads to the expression of a distinct set of *Wnt* genes in two different contexts. Together with previous data on Gli function, our present results suggest that in normal development, Gli proteins induce cell differentiation (e.g., [1, 2]), proliferation (e.g., [27, 30]), and morphogenesis, and that at least the latter requires Wnt function. Gli and Wnt factors could, therefore, contribute to tumorigenesis by altering the proliferative and morphogenetic properties of precursor cells. Finally, since Gli2/3 respond to and mediate FGF signaling [5] and Gli1/2 respond to and mediate SHH signaling (e.g., [1, 2, 4]), the regulation of *Wnt* gene expression by Gli proteins raises the possibility that these could link FGF and/or HH signaling with Wnt function.

Supplementary material

Methods for microinjection, RT-PCR assays, in situ hybridization, and histochemistry can be found with the electronic version of this article at http://images.cellpress.com/supmat/supmatin.htm.

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