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RESEARCH ARTICLE

BMP-mediated induction of GATA4/5/6 blocks somitic responsiveness to SHH

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ABSTRACT

The relative timing of SHH and BMP signals controls whether presomitic mesoderm (PSM) cells will adopt either a chondrogenic or lateral plate mesoderm fate. Here we document that SHH-mediated induction of *Nkx3.2* maintains the competence of somitic cells to initiate chondrogenesis in response to subsequent BMP signals by repressing BMP-dependent induction of GATA genes. Conversely, administration of BMP signals to PSM or forced expression of GATA family members in chick PSM explants blocks induction of hedgehog-dependent gene expression. We demonstrate that GATA factors can interact with Gli factors and can recruit the transcriptional co-factor FOG1 (ZFPM1) to the regulatory region of the mouse *Gli1* gene, repressing the induction of *Gli1* by SHH by binding to both GATA and Gli binding sites. Knockdown of FOG1 reverses the ability of GATA factors to repress *Gli1* expression. Our findings uncover a novel role for GATA transcription factors as repressors of hedgehog signaling, and document that NKX3.2 maintains the ability of sclerotomal cells to express SHH transcriptional targets in the presence of BMP signals by repressing the induction of *Gata4*/*5*/*6*.

KEY WORDS: NKX3.2, GATA factors, SHH, SOX9, Chondrogenesis, Chicken, Mouse

INTRODUCTION

The majority of the vertebrate skeleton arises by the process of endochondral ossification, in which each skeletal element is established and patterned as cartilage, later to be replaced by bone. The cartilage of the limb derives from the lateral plate mesoderm, whereas the vertebrae and ribs derive from the ventral compartment of the somites termed the sclerotome. Prior work of several laboratories has established that varying levels of BMP signals specify the fate of nascent mesodermal cells, with low levels of BMP signaling necessary for somite induction and higher levels of BMP signaling necessary for the formation of lateral plate mesoderm (reviewed by James and Schultheiss, 2005). However, once cells are specified as paraxial mesoderm, sonic hedgehog (SHH) emitted by the notochord and floor plate of the neural tube alters the response of the adjacent somitic cells to subsequent BMP signaling, such that now BMP signals induce chondrogenesis as opposed to lateral plate mesoderm identity (Murtaugh et al., 1999). This effect can be observed *in vitro*, as treatment of chicken presomitic mesoderm (PSM) explants with SHH for 2 days, followed by BMP2 administration, induces the robust expression of chondrogenic differentiation markers such as SOX9, aggrecan and collagen IX (Murtaugh et al., 1999). By contrast, simultaneous exposure of PSM explants to both SHH and BMP signals fails to induce chondrogenesis, but rather induces lateral plate gene expression (Murtaugh et al., 1999). In the present work, we investigate how the relative timing of SHH and BMP signals in PSM leads to such different cellular outcomes.

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RESULTS

Nkx3.2 and GATA genes display reciprocal patterns of expression *in vitro* and *in vivo*

We have previously found that chondrogenesis in explants of chicken PSM is robustly induced by sequential exposure to SHH followed by BMP4 (Murtaugh et al., 1999). In striking contrast, simultaneous administration of both SHH and BMP to chicken PSM explants completely blocks chondrogenesis in such cultures (Murtaugh et al., 1999). Because BMP signals are known to lateralize paraxial mesoderm into lateral plate tissue (reviewed by James and Schultheiss, 2005), we analyzed the expression of the lateral plate mesoderm markers GATA4, GATA5 and GATA6 (GATA4/5/6) in chicken PSM explants that had been exposed to SHH and BMP4 either simultaneously or sequentially. Sequential exposure of PSM explants to SHH added at the beginning of the culture period, followed 2 days later by BMP4, induced robust expression of the chondrogenic markers aggrecan, epiphycan and collagen IX after a total of 5 days of culture (Fig. 1A, lane 1). By contrast, simultaneous exposure of such explants to both SHH and BMP4 for the entire culture period completely blocked chondrogenesis in such cultures and instead induced expression of the lateral plate markers *Gata4/5/6* (Fig. 1A, lane 2).

Nkx3.2 is expressed in chicken PSM explants following sequential administration of SHH and BMP signals (Murtaugh et al., 2001), a signaling regimen that blocks BMP-mediated induction of *Gata4/5/6* (Fig. 1A). Conversely, *Gata4/5/6* are expressed in chicken PSM explants that have been simultaneously exposed to both BMP4 and SHH signals (Fig. 1A), a signaling regimen that blocks SHH-mediated induction of *Nkx3.2* (Murtaugh et al., 1999). Thus, *Nkx3.2* and *Gata4/5/6* are reciprocally expressed in chicken PSM explants following sequential or simultaneous exposure to SHH and BMP4. To determine whether NKX3.2 and GATA family members also display a reciprocal expression pattern

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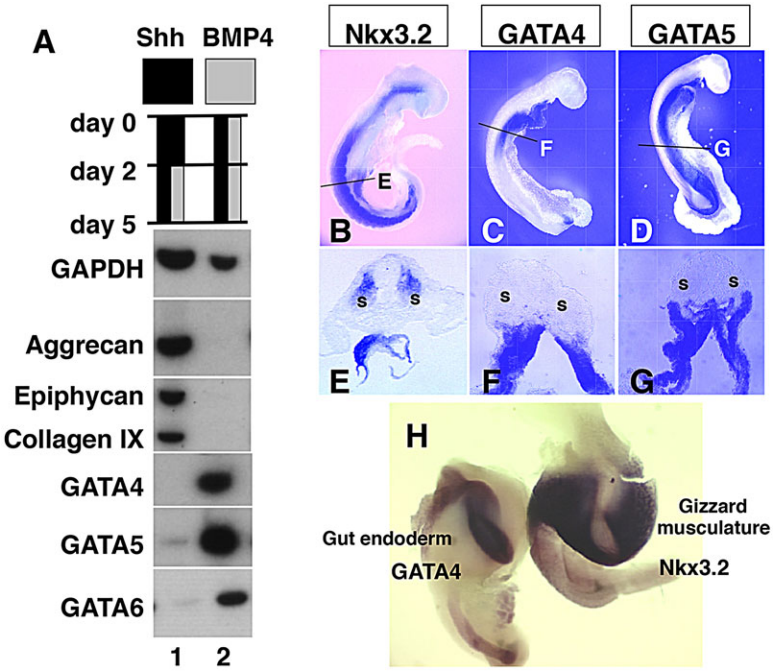

Fig. 1. *Nkx3.2* and GATA genes display mutually exclusive expression patterns *in vitro* and *in vivo*. (A) Chicken presomitic mesoderm (PSM) was cultured in the presence of SHH and BMP4 for the indicated time period, and gene expression was assayed by RT-PCR (n=4). (B-D) WISH analysis of HH stage 12-13 chicken embryos. (E-G) Sections of embryos in B-D. (H) WISH analysis for *Nkx3.2* and *Gata4* in HH stage 30 chicken embryos. s, somite.

in vivo, we examined their expression by whole-mount *in situ* hybridization (WISH) in Hamburger–Hamilton (HH) stage 10 to 14 chicken embryos. Interestingly, expression of these two classes of genes is nearly mutually exclusive in the mesoderm, with *Nkx3.2* being restricted to the sclerotome of the paraxial mesoderm, whereas *Gata4* and *Gata5* are expressed in the lateral plate mesoderm (Fig. 1B-G). Note, however, that *Nkx3.2* is in addition expressed in a restricted region of the left lateral plate mesoderm (Fig. 1B,E). Subsequent expression of these genes is also non-overlapping in the endoderm and surrounding mesoderm, with *Gata4* expression restricted to the endodermal gut tube and *Nkx3.2* present in the surrounding mesoderm of the gizzard in HH stage 30 chicken embryos (Fig. 1H).

BMP4-mediated repression of SHH signal transduction correlates with the induction of GATA family members

The administration of BMP2/4 to PSM explants at the start of their culture period has been shown to block both myogenesis [in response to simultaneous SHH and Wnt signals (Reshef et al., 1998)] and chondrogenesis [in response to sequential SHH and BMP signals (Murtaugh et al., 1999)]. In both these instances SHH signals are necessary to promote lineage specification. Thus, we speculated that simultaneous administration of BMP2/4 might act to attenuate SHH signaling. To examine this issue, we cultured chicken PSM explants in SHH alone or in the combination of SHH plus BMP4. In the latter case, we administered BMP4 1 day prior to adding SHH to the cultures. When administered alone, SHH markedly induced the expression of *Gli1*, *Sox9* and *Nkx3.2* (Fig. 2, lane 3). By contrast, prior culture of PSM explants in BMP4 severely attenuated (*Gli1*) or eliminated (*Sox9* and *Nkx3.2*) the induction of these hedgehog-responsive genes (Fig. 2, lane 4). Repression of the hedgehog transcriptional targets correlated with the induced expression of *Gata4/5/6* (Fig. 2, lane 4). Interestingly, expression of *Sox9* and *Nkx3.2* is not repressed by BMP signals following prior exposure of PSM explants to SHH (Zeng et al., 2002), conditions that fail to induce the expression of *Gata4/5/6*.

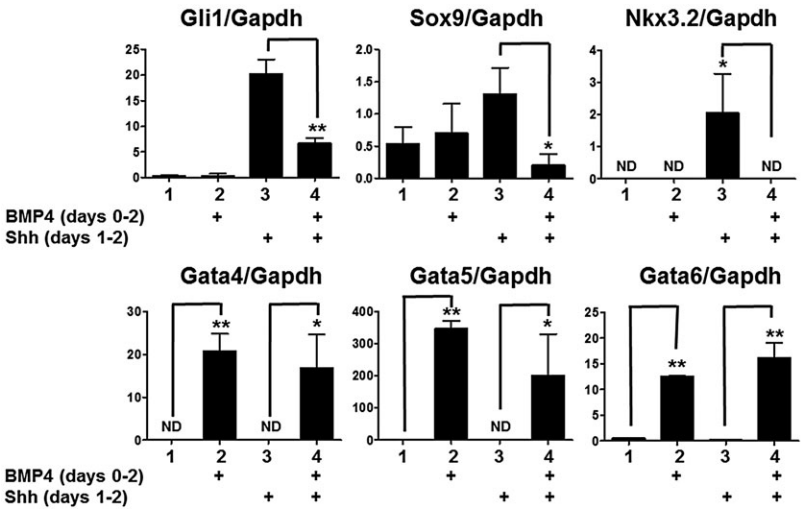

Fig. 2. BMP4-mediated repression of SHH signal transduction correlates with induction of GATA family members. Chicken PSM was cultured in the presence of SHH and BMP4 for the indicated time period, and gene expression was assayed by RT-qPCR. Expression was normalized to that of *Gapdh*. *P≤0.05, **P≤0.001, Student's t-test (n=4). Error bars indicate s.d.

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Thus, BMP-mediated repression of hedgehog target genes in PSM explants correlates with the induction of GATA factors.

Ectopic expression of GATA factors can repress the induction of SHH-responsive genes in PSM explants

Because BMP-induced expression of GATA4/5/6 correlated with the repression of hedgehog transcriptional targets, we postulated that these GATA family members might somehow attenuate hedgehog signal transduction. To investigate this possibility, we determined the effect of exogenous GATA4 in PSM cultured under chondrogenesis-inducing conditions. We infected chicken PSM explants with avian retroviruses encoding GFP (RCAS-GFP) or GATA4 (RCAS-GATA4) and cultured the explants in SHH for 2 days followed by BMP4 for an additional 3 days (Fig. 3A). Expression of the SHH transcriptional targets patched 1, *Sox9* and *Nkx3.2* was significantly dampened in RCAS-GATA4-infected cultures and expression of the chondrocyte differentiation markers aggrecan and epiphycan was extinguished (Fig. 3A).

In addition, we assayed whether exogenous GATA5 would similarly attenuate induction of SHH-responsive genes in PSM explants. Infection of PSM explants with RCAS-GATA5 dramatically decreased SHH-mediated induction of patched 1, patched 2, *Gli1*, *Nkx3.2* and *Sox9* in these cultures (Fig. 3B). Interestingly, the repression of these various hedgehog-responsive genes displayed differential sensitivity to GATA5 expression. Induction of *Sox9* and *Nkx3.2* was blocked by ectopic expression of GATA5 in PSM explants cultured in low or high levels of SHH. By contrast, induction of *Gli1*, patched 1 and patched 2 was most effectively blocked by ectopic expression of GATA5 in PSM explants cultured in relatively low levels of SHH. Together, these findings indicate that GATA4 or GATA5 can attenuate hedgehog signal transduction in chicken PSM explants and thus mimic the effect of BMP4 administration to this tissue.

NKX3.2 represses BMP-mediated induction of GATA family members

Our findings indicate that induction of GATA transcription factors in PSM explants somehow blocks SHH-induced expression of SOX9 and NKX3.2, two crucial chondrogenic regulators. Because *Nkx3.2* and *Gata4*/5/6 are frequently expressed in a mutually exclusive pattern *in vivo* (Fig. 1B-H), and NKX3.2 is a transcriptional repressor (Murtaugh et al., 2001), we examined whether NKX3.2 might conversely repress the expression of GATA4/5/6. Chicken PSM explants were infected with RCAS-GFP or RCAS-Nkx3.2. After 2 days of culture, BMP2 was added to the medium and the explants were cultured for an additional 3 days. PSM explants infected with RCAS-GFP robustly expressed *Gata4/5/6* and did not express aggrecan or epiphycan, two markers of chondrocyte differentiation (Fig. 4A, lane 1). By contrast, PSM explants infected with RCAS-Nkx3.2 expressed high levels of aggrecan and epiphycan, and significantly decreased levels of *Gata4/5/6* (Fig. 4A, lane 2). Thus, exogenous NKX3.2 can potently block BMP-induced expression of GATA factors while simultaneously promoting the expression of chondrogenic markers in PSM explants. Interestingly, we noted that exogenous NKX3.2 increased the expression of *Gli1* in PSM explants (Fig. 4A, lane 2), suggesting that NKX3.2 represses the expression of a repressor(s) of hedgehog signaling. Although inclusion of the smoothened antagonist cyclopamine attenuated the induction of both the chondrogenic markers and *Gli1* by NKX3.2, it did not blunt the repression of GATA factors by NKX3.2 (Fig. 4A, lanes 3 and 4).

SMAD1/4 interaction with NKX3.2 has been demonstrated to recruit the Sin3A co-repressor complex to *Nkx3.2* in a BMP-dependent fashion (Kim and Lassar, 2003). Thus, it is formally possible that NKX3.2 blocks GATA gene expression by titrating SMAD1/4 away from other transcription factors that are necessary to induce GATA gene expression. To investigate this possibility we employed a mutant form of NKX3.2, Nkx3.2-NQ, which cannot bind to DNA but can still interact with SMAD1/4 and the Sin3A co-repressor complex (Kim et al., 2003). In contrast to RCAS-Nkx3.2-WT, RCAS-Nkx3.2-NQ failed to induce chondrogenesis and to block the induction of *Gata4* by BMP4 in infected PSM cultures (Fig. 4B). Thus, DNA interaction is essential for NKX3.2 to induce chondrogenesis and to block BMP-mediated induction of GATA4 expression.

NKX3.2 induces chondrogenesis in PSM explants by acting as a transcriptional repressor (Murtaugh et al., 2001). Indeed, a reverse-function form of NKX3.2, Nkx3.2-VP16, which contains the VP16 transcriptional activation domain and is thus a potent

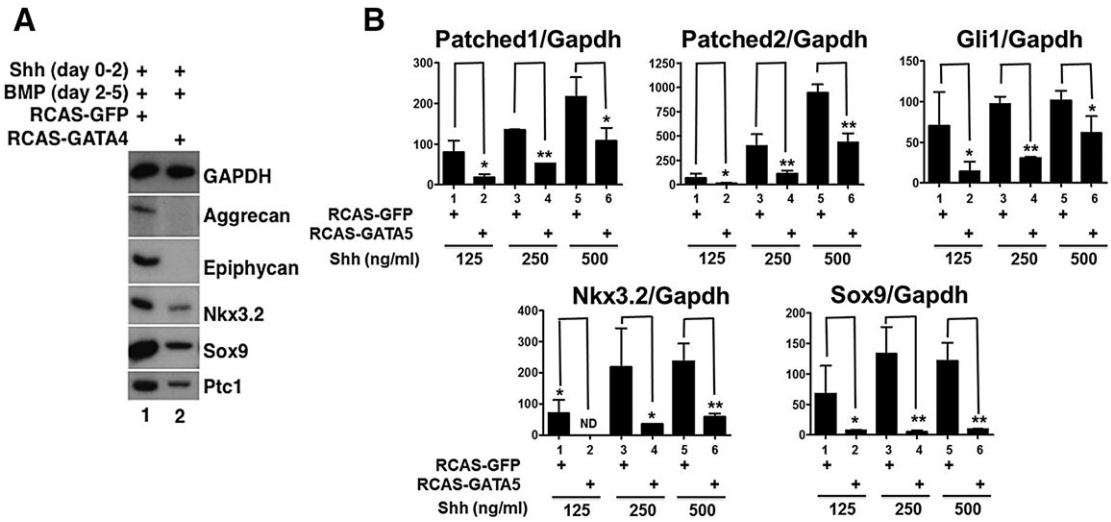

Fig. 3. GATA factors can repress the induction of SHH-responsive genes in PSM explants. Chicken PSM was infected with RCAS-GFP or RCAS-GATA4 (A) or RCAS-GATA5 (B), cultured in the presence of SHH and BMP4 for the indicated time period, and gene expression assayed by RT-PCR (A) or RT-qPCR (B). In B, expression was normalized to that of *Gapdh*. **P*≤0.05, ***P*≤0.001, Student's *t*-test. Error bars indicate s.d. *n*=2 (A,B).

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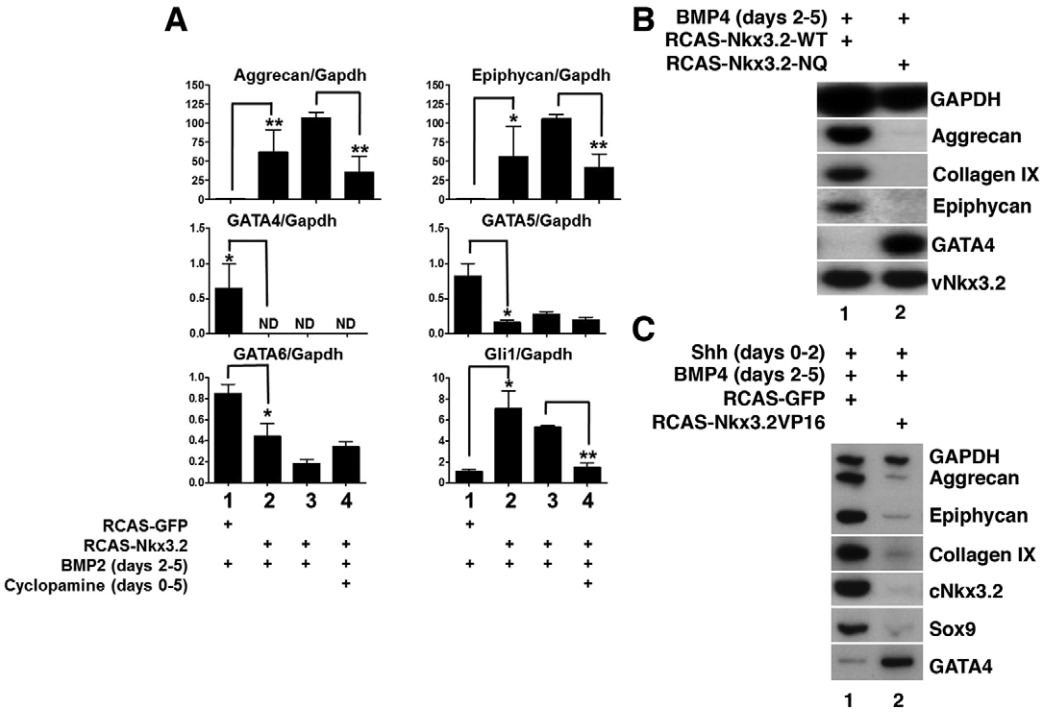

Fig. 4. NKX3.2 represses BMP-mediated induction of GATA family members. (A,B) Chicken PSM was infected with either RCAS-GFP, RCAS-Nkx3.2-WT or RCAS-Nkx3.2-NQ, cultured in the presence of BMP2/4 and cyclopamine as indicated, and gene expression assayed by RT-qPCR (A) or RT-PCR (B). Virus-encoded WT or mutant *Nkx3.2* transcript levels are indicated as vNkx3.2. In A, PSM taken from opposite sides of the same embryos (i.e. left versus right PSM) was cultured in lanes 1 and 2 and in lanes 3 and 4. Expression was normalized to that of *Gapdh*. **P*≤0.05, ***P*≤0.001, Student's *t*-test (*n*=4). Error bars indicate s.d. (C) PSM explants were infected with RCAS-GFP or RCAS-Nkx3.2-VP16, cultured in the presence of SHH and BMP4 as indicated, and gene expression was assayed by RT-PCR (*n*=2).

transcriptional activator (Murtaugh et al., 2001) can block somitic chondrogenesis in response to sequential SHH and BMP signals (Zeng et al., 2002). Because NKX3.2 represses BMP-mediated induction of GATA factors in PSM explants, we investigated whether a reverse-function form of NKX3.2 would have the opposite effect on GATA gene expression. PSM explants were infected with RCAS-GFP or RCAS-Nkx3.2-VP16. In this case, the explants were sequentially cultured in medium containing SHH for 2 days followed by medium containing BMP4 for an additional 3 days. Whereas explants infected with RCAS-GFP displayed high-level expression of chondrogenic markers and only trace levels of *Gata4* (Fig. 4C, lane 1), those infected with RCAS-Nkx3.2-VP16 lacked significant expression of chondrogenic markers but instead expressed elevated levels of *Gata4* (Fig. 4C, lane 2). Thus, a reverse-function form of NKX3.2 (which induces the expression of transcriptional targets that are usually repressed by NKX3.2) blocks somitic chondrogenesis while coordinately maintaining the expression of GATA4 in response to BMP cues.

Taken together, these findings suggest that SHH-mediated induction of NKX3.2 expression in the sclerotome serves to promote chondrogenesis and to maintain the ability of this tissue to respond to further hedgehog signals by blocking BMP-mediated induction of GATA4/5/6. Repression of GATA4/5/6 expression by NKX3.2 requires both DNA binding and an intact C-terminal transcriptional repression domain, suggesting that NKX3.2 directly or indirectly represses the expression of these GATA family members.

GATA factors block induction of Gli transcriptional targets downstream of smoothened activity

Because we found that GATA factors could block the induction of hedgehog-responsive genes in explants of embryonic tissue, we investigated whether transfection of GATA4/5/6 would repress SHH-mediated induction of a luciferase reporter driven by reiterated Gli binding sites [8×3′-Gli-BS-Luciferase (Dai et al., 1999)]. Indeed, co-transfection of murine 10T1/2 cells with either GATA4, GATA5 or GATA6 repressed SHH-mediated induction of 8×3′-Gli-BS-Luciferase in a dose-dependent fashion (Fig. 5A), indicating that GATA factors can block hedgehog signaling in this tissue culture system. As it was possible that GATA factors could be blocking hedgehog signaling either upstream or downstream of smoothened translocation into the primary cilia (Rohatgi et al., 2007), we assayed whether GATA4 could repress induction of 8×3′-Gli-BS-Luciferase by an activated form of smoothened (Smo-M2), the smoothened agonist SAG [which induces smoothened translocation into the primary cilia (Rohatgi et al., 2007)], or by co-transfected GLI1 (which activates hedgehog target genes independently of the primary cilia). We found that co-transfected GATA4 could efficiently block activation of 8×3′-Gli-BS-Luciferase by either SHH, Smo-M2, SAG or GLI1 (Fig. 5B). These findings indicate that GATA factors can block hedgehog signaling downstream of smoothened translocation into the primary cilia.

To further investigate whether GATA factors block hedgehog signaling downstream of smoothened, we examined whether GATA factors would block Gli transcriptional targets in *Sufu*-deficient mouse embryo fibroblasts ($Sufu^{-/-}$ MEFs) (Svard et al., 2006). In the absence of hedgehog signaling, SUFU tethers full-length GLI2 (Gli2FL) and full-length GLI3 (Gli3FL) in the cytoplasm (Humke et al., 2010; Tukachinsky et al., 2010) and is necessary for processing these proteins into transcriptional repressors (Humke et al., 2010). Thus, in cells lacking *Sufu*, even in the absence of hedgehog ligands, Gli2FL and Gli3FL are constitutively present in

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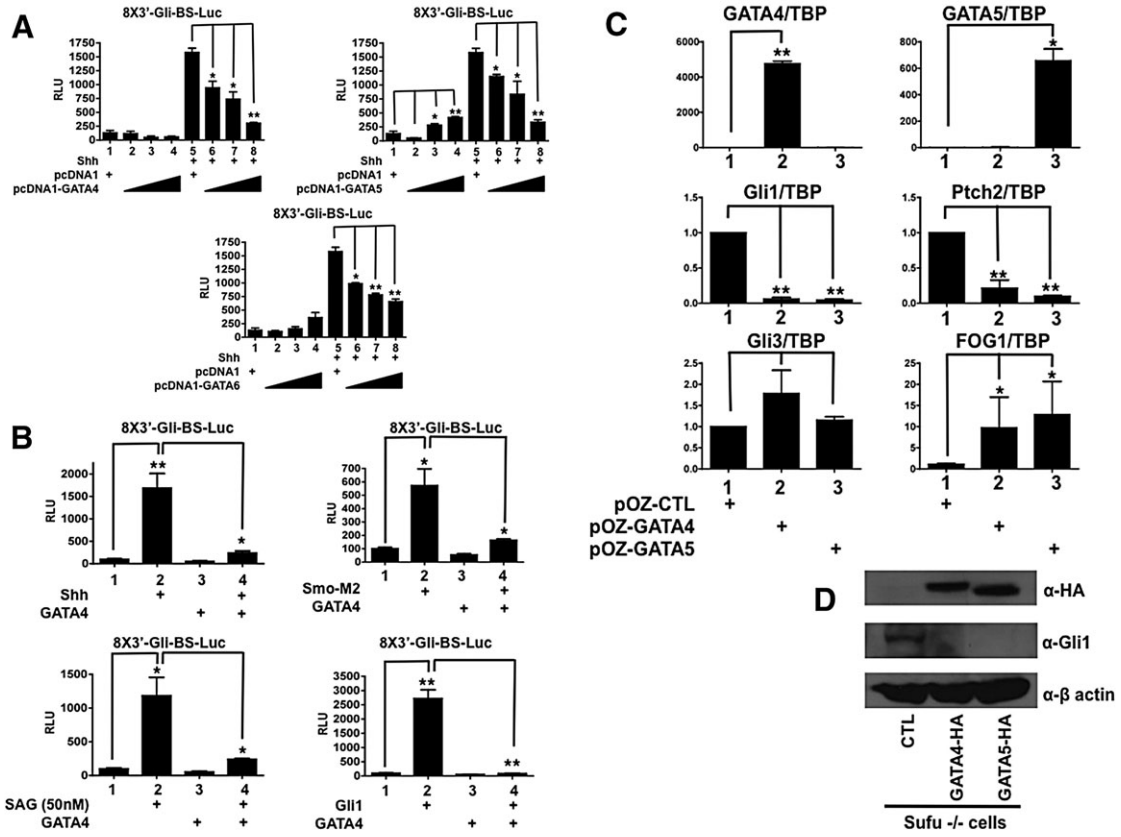

Fig. 5. GATA factors block induction of Gli transcriptional targets downstream of smoothened activity. (A) Murine 10T1/2 cells were transfected with 8×3′-Gli-BS-firefly luciferase, SV40 renilla luciferase (as a control) and expression vehicles for SHH and GATA4/5/6 as indicated. Relative luciferase units (RLU) are displayed. (B) Murine NIH 3T3 cells were transfected with 8×3′-Gli-BS-firefly luciferase, SV40 renilla luciferase (control) and expression vehicles for SHH, Smo-M2, GLI1 and GATA4 as indicated and/or cultured in 50 nM smoothened agonist (SAG). RLU are displayed. (C,D) Gene expression in polyclones of *Sufu*-deficient MEFs infected with either pOZ-HA (parental retrovirus), pOZ-GATA4-HA or pOZ-GATA5-HA was analyzed by RT-qPCR (C) or western blot (D). In C, expression was normalized to that of TATA binding protein (*Tbp*). $^{*}P \leq 0.05$, $^{**}P \leq 0.001$, Student's *t*-test. Error bars indicate s.d. $n=2$ (A,B) or $n=3$ (C).

the nucleus, where they activate the expression of Gli transcriptional targets (Humke et al., 2010). We found that the expression of *Gli1* and patched 2 mRNA was markedly diminished in *Sufu*$^{-/-}$ MEFs programmed to express retrovirus-encoded GATA4 or GATA5 (Fig. 5C, lanes 2 or 3), and that the expression of GLI1 protein was similarly repressed in *Sufu*$^{-/-}$ MEFs programmed to express these GATA factors (Fig. 5D). By contrast, expression of *Gli3* in *Sufu*$^{-/-}$ MEFs was not significantly altered by ectopic expression of GATA4 or GATA5 (Fig. 5C). Because smoothened function is not necessary for activation of hedgehog targets in *Sufu*$^{-/-}$ MEFs (Svard et al., 2006), these findings confirm our previous observations (Fig. 5B) that GATA factors repress the expression of hedgehog target genes downstream of smoothened activation.

GATA factors can indirectly bind to Gli binding sites via protein:protein interaction

Because GATA factors can repress Gli-dependent activation of transcriptional targets, we investigated whether GATA factors can bind, directly or indirectly, to a Gli binding site. We first co-transfected murine NIH 3T3 cells with 8×3′-Gli-BS-Luciferase plus either the GATA4 zinc-finger domain (GATA4 ZF) or a fusion protein consisting of the GATA4 zinc-finger domain appended to the Herpes virus VP16 transcriptional activation domain (GATA4-ZF-VP16) (schematized in supplementary material Fig. S1). Interestingly, GATA4-ZF-VP16 robustly activated the expression of 8×3′-Gli-BS(WT)-Luciferase, which contains WT Gli binding sites, but failed to activate 8×3′-Gli-BS(mutant)-Luciferase, which contains mutant Gli binding sites (Fig. 6A). Thus, GATA4-ZF-VP16-mediated induction of 8×3′-Gli-WT-BS-Luciferase requires intact Gli binding sites.

We speculated that GATA4-ZF-VP16 might directly bind to Gli binding sites in 8×3′-Gli-WT-BS-Luciferase or indirectly associate with these sites by protein:protein interaction. To test the former possibility we made nuclear extracts from COS-7 cells transfected with either GATA4, GATA5, GATA6 or GLI1. Although nuclear extracts made from cells transfected with GATA4, GATA5 or GATA6 displayed readily detectable GATA site binding activity, these extracts failed to display any Gli site binding activity (Fig. 6B). Conversely, nuclear extracts made from GLI1-transfected cells displayed robust Gli site binding activity but no GATA site binding activity (Fig. 6B). These findings indicate that GATA factors do not bind directly to Gli binding sites, and thus suggest that GATA4-ZF-VP16 might activate the expression of 8×3′-Gli-BS(WT)-Luciferase by indirectly binding to Gli binding sites in this reporter via protein-protein interaction.

GATA4 can interact with Gli factors

To investigate whether there is protein:protein interaction between GATA and Gli factors we employed a mammalian two-hybrid assay. NIH 3T3 cells were co-transfected with a luciferase reporter driven by reiterated Gal4 binding sites (GAL4-luciferase) plus expression vehicles encoding the Gal4 DNA-binding domain alone

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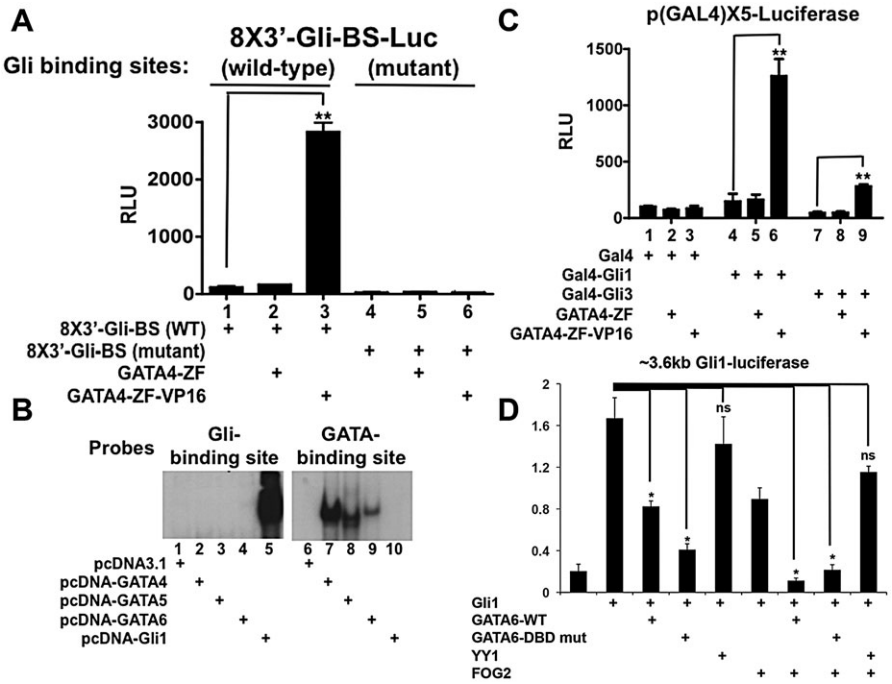

Fig. 6. GATA and Gli factors associate by protein:protein interaction. (A) NIH 3T3 cells were transfected with 8×3′-Gli-BS(WT)-Luciferase containing WT GLI1 binding sites (lanes 1-3) or 8×3′-Gli-BS(mutant)-Luciferase containing mutated GLI1 binding sites (lanes 4-6), SV40 renilla luciferase (as a control) and expression vehicles encoding the GATA4 zinc-finger domain (GATA-ZF) or the GATA4 zinc-finger domain appended to the VP16 transcriptional activation domain (GATA4-ZF-VP16). RLU are displayed. (B) EMSA analysis of Gli and GATA binding. COS-7 cells were transfected with the indicated expression vehicles. Nuclear extracts from these cells were incubated with radiolabeled oligos carrying a Gli binding site located upstream of the *Gli1* promoter (Dai et al., 1999) (lanes 1-5) or a GATA binding site located upstream of the *Nkx2.5* promoter (Lee et al., 2004) (lanes 6-10). (C) NIH 3T3 cells were co-transfected with GAL4-firefly luciferase and SV40 renilla luciferase (control) plus expression vehicles encoding the Gal4 DNA-binding domain alone (lanes 1-3) or the Gal4 DNA-binding domain fused to GLI1 (lanes 4-6) or Gli3FL (lanes 7-9) in the absence or presence of GATA4-ZF-VP16 or GATA4-ZF. RLU are displayed. (D) NIH 3T3 cells were co-transfected with a ~3.6 kb Gli1-firefly luciferase reporter plus expression vehicles encoding either GLI1, GATA6-WT, GATA6-DBDmut, YY1 or FOG2, as indicated. Firefly luciferase units were normalized to the expression of co-transfected SV40-renilla luciferase to obtain RLU. *$P \leq 0.05$, **$P \leq 0.001$, Student's *t*-test (ns, not significant). Error bars indicate s.d. $n=2$ (A,D) or $n=3$ (C).

or the Gal4 DNA-binding domain fused to GLI1 (Gal4-Gli1) or Gli3FL (Gal4-Gli3FL). To assay for Gli:GATA interaction, the cells were also transfected with expression vehicles encoding GATA4-ZF-VP16 or GATA4-ZF. Co-transfection of GATA4-ZF-VP16 induced the expression of GAL4-luciferase in the presence of Gal4-Gli1 or Gal4-Gli3FL, but not in the presence of the unfused Gal4 DNA-binding domain (Fig. 6C). These findings indicate that Gal4-Gli1 or Gal4-Gli3FL can recruit GATA4-ZF-VP16 to activate the expression of GAL4-luciferase, implying that Gli proteins and the zinc-finger domain of GATA factors can associate, directly or indirectly, via protein:protein interaction.

GATA6 can repress the expression of a *Gli1* promoter-luciferase reporter in the absence of direct DNA interaction

Our findings suggested that GATA factors can be recruited to Gli binding sites via protein:protein interaction with Gli proteins. Thus, we investigated whether direct interaction of GATA factors with DNA is necessary for these factors to repress *Gli1* expression, which itself is known to be regulated by Gli transcription factors (Dai et al., 1999). We had previously found that co-transfection of GATA6 plus FOG2 (ZFPM2) would synergistically repress the induction of a ~3.6 kb Gli1-luciferase reporter by co-transfected GLI1 in NIH 3T3 cells (Kozhemyakina et al., 2014). We found that a mutant form of GATA6 that cannot directly bind to DNA [GATA6-DBDmut (Kozhemyakina et al., 2014)] could, when co-transfected together with FOG2, efficiently repress the induction of ~3.6 kb Gli1-luciferase by GLI1 (Fig. 6D), indicating that GATA6 can repress the expression of this reporter in the absence of direct DNA interaction. Importantly, GATA6-WT and GATA6-DBDmut are expressed at approximately equivalent levels in transfected NIH 3T3 cells (supplementary material Fig. S2). We previously noted that, unlike GATA6-WT, GATA6-DBDmut is unable to repress the expression of the *Shh* limb bud enhancer (which contains GATA but not Gli binding sites) (Kozhemyakina et al., 2014), suggesting that GATA6-DBDmut cannot directly bind to DNA.

To evaluate whether other zinc-finger transcription factors would similarly repress the induction of ~3.6 kb Gli1-luciferase by GLI1, we co-transfected this reporter together with YY1. In contrast to GATA6, YY1 failed to significantly repress the induction of ~3.6 kb Gli1-luciferase by GLI1 in either the absence or presence of FOG2 (Fig. 6D). Taken together, these findings suggest that GATA factors (and not all zinc-finger transcription factors) can repress expression of the *Gli1* promoter in the absence of direct DNA interaction.

GATA factors bind to both GATA and Gli binding sites in the *Gli1* regulatory region *in vivo*, and can recruit FOG1 to these sequences

Because the ectopic expression of GATA4 or GATA5 efficiently blocked *Gli1* expression in *Sufu*-deficient cells, we investigated whether these GATA factors might directly bind to *Gli1* regulatory regions in *Sufu*$^{-/-}$ cells programmed to express these GATA factors. Chromatin immunoprecipitation (ChIP) of GATA4 or GATA5

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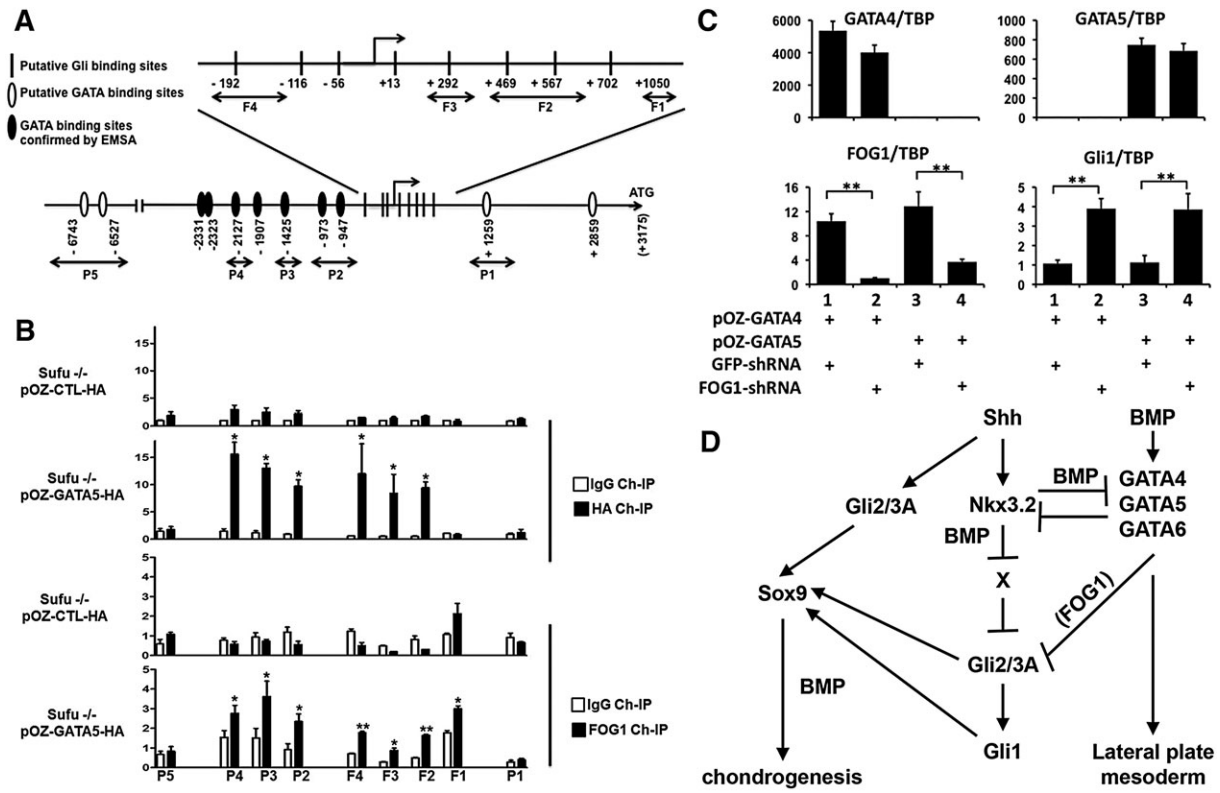

Fig. 7. GATA factors bind to both GATA and Gli binding sites surrounding the *Gli1* promoter. (A) Schematic of the mouse *Gli1* gene showing putative Gli binding sites [identified by Dai et al. (1999)] and GATA binding sites (those confirmed by EMSA with *in vitro* translated GATA6 are depicted as black ovals). (B) ChIP for HA or FOG1-associated sequences in polyclones of *Sufu*$^{-/-}$ MEFs infected with pOZ-HA (parental retrovirus) or pOZ-GATA5-HA, employing the primers depicted in A. (C) *Sufu*$^{-/-}$ MEFs programmed to express GATA4 or GATA5 were infected with lentiviruses encoding shRNAs targeting GFP or FOG1, and gene expression was assayed by RT-qPCR. Expression of the various genes was normalized to that of *Tbp*. **P*≤0.05, ***P*≤0.001, Student's *t*-test. Error bars indicate s.d. *n*=2 (B, FOG1) or *n*=3 (B, HA; C). (D) SHH induces the expression of *Nkx3.2* in the sclerotome, which both blocks the induction of *Gata4*/*5*/*6* by subsequent BMP signals and represses the expression of a repressor of hedgehog signaling (termed 'X'). Conversely, BMP-mediated induction of *Gata4*/*5*/*6* dampens the response of mesodermal cells to hedgehog signaling (in a FOG-dependent fashion) and consequently blocks SHH-mediated induction of *Nkx3.2* and *Sox9*.

indicated that both bind to chromatin encompassing GATA or Gli binding sites located between −2.3 kb and +700 bp relative to the *Gli1* transcriptional start site (TSS) (Fig. 7A,B; data not shown). By contrast, neither GATA factor bound to chromatin encompassing putative GATA binding sites that are located more distally from the *Gli1* TSS (Fig. 7A,B; data not shown).

Prior work has established that eight Gli binding sites that are proximal to the *Gli1* TSS (extending from −243 to +750) can confer responsiveness to hedgehog signaling and serve as binding sites for GLI3 (Dai et al., 1999). Interestingly, GATA5 associated equally well with chromatin containing either GATA binding sites (located at approximately −2 kb to −1 kb) or Gli binding sites (located at approximately −200 bp to +700 bp) (Fig. 7A,B). To ensure that GATA factor association with the promoter-proximal Gli binding sites reflected interaction with these sequences (as opposed to interaction with more distant GATA binding sites) the chromatin was sheared to 200-400 bp prior to immunoprecipitation. In addition, EMSA employing *in vitro* translated GATA factor with tiled oligos encompassing all sequences from −379 bp to +781 bp revealed no detectable direct GATA binding sites in this promoter-proximal region that contained the reiterated Gli binding sites (data not shown). These findings suggest that GATA factors bind to the *Gli1* regulatory region by both protein:DNA interaction (on GATA sites) and protein:protein interaction (on Gli sites).

How might GATA factor association with the *Gli1* regulatory region act to repress expression of GLI1? GATA factors are either transcriptional activators or repressors (reviewed by Chlon and Crispino, 2012). GATA factor transcriptional repressor activity requires interaction with either of the transcriptional co-factors FOG1 (ZFPM1) or FOG2 (which in turn recruit the co-repressors NuRD and CtBP). Interestingly, we found that forced expression of GATA4 or GATA5 induced the expression of *Fog1* in *Sufu*-deficient MEFs and NIH 3T3 cells, and of *Fog2* in NIH 3T3 cells (Fig. 5C; supplementary material Figs S3 and S4). In addition, forced expression of GATA5 (in *Sufu*-deficient MEFs) recruited FOG1 to chromatin containing either GATA or Gli binding sites that encompass the *Gli1* TSS (Fig. 7B; data not shown).

Knockdown of FOG1 reverses the ability of GATA factors to repress expression of *Gli1*

To examine whether GATA-mediated recruitment of FOG1 to the *Gli1* promoter is necessary for GATA factors to repress the expression of *Gli1* in *Sufu*$^{-/-}$ MEFs, we knocked down FOG1 expression in *Sufu*-deficient MEFs programmed to express GATA4 or GATA5. The expression of *Fog1* RNA and the 160 kDa FOG1 protein (Snow et al., 2010) was significantly decreased in polyclones of cells programmed to express an shRNA targeting this mRNA (Fig. 7C; supplementary material Fig. S3). Interestingly, shRNA-mediated knockdown of FOG1 significantly boosted *Gli1* expression ~4-fold

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in these cells (Fig. 7C, lanes 2 and 4), suggesting that loss of FOG1 relieved GATA-mediated inhibition of *Gli1* expression.

Taken together, these findings indicate that GATA factors repress the expression of hedgehog targets, such as *Gli1*, by recruiting FOG co-factors to GATA and/or Gli binding sites in the regulatory regions that drive the expression of these genes.

DISCUSSION

NKX3.2 blocks the expression of three inhibitors of hedgehog signaling: GATA4/5/6

SHH signals from the notochord and floor plate of the neural tube to induce somitic cells to express hedgehog-responsive genes particular to the sclerotome (*Sox9*, *Nkx3.2* and *Pax1*) and non-tissue-restricted hedgehog-responsive genes (*Gli1* and patched 1/2). One of the consequences of the induction of *Nkx3.2* and *Sox9* in the sclerotome is that this tissue becomes competent to initiate the chondrocyte differentiation program in response to subsequent BMP signals (Zeng et al., 2002). In this study, we demonstrate that NKX3.2 blocks the induction of GATA transcription factors in chicken PSM explants, and that GATA4/5/6 in turn block the induction of hedgehog-responsive genes (including *Sox9*). Thus, by inhibiting BMP-mediated induction of *Gata4/5/6*, NKX3.2 maintains the ability of sclerotomal cells to undergo chondrogenesis and to respond to further hedgehog signals (outlined in Fig. 7D). Because GATA transcription factors are necessary for BMP signals to induce endothelial cell formation in somitic tissue (Kamei et al., 2011), it seems plausible that NKX3.2 expression in the sclerotome (and consequent repression of GATA gene induction) blocks aberrant vasculogenesis in the progeny of sclerotomal cells.

NKX3.2 and GATA factors engage in reciprocal repression

By varying the timing of chicken PSM explant exposure to SHH and BMP, we noted that *Nkx3.2* and *Gata4/5/6* are reciprocally expressed in explants of paraxial mesoderm induced to become either chondrogenic or lateral plate tissue. Because reciprocal expression of transcriptional regulators often reflects mutually repressing negative-feedback loops (e.g. Briscoe et al., 2000), we explored whether NKX3.2 and GATA factors mutually repress one another's expression. Indeed, ectopic expression of NKX3.2 in paraxial mesoderm explants blocked GATA gene induction by BMP signals and, conversely, ectopic expression of GATA factors in this same tissue blocked *Nkx3.2* induction by SHH (as outlined in Fig. 7D). In addition to blocking the expression of GATA transcription factors, we noted that NKX3.2 expression increased the expression of *Gli1* in PSM explants, suggesting that NKX3.2 represses the expression of a repressor(s) of hedgehog signaling (summarized in Fig. 7D). Thus, GATA factors and NKX3.2 set up two distinct ground states in which mesodermal cells are either relatively unresponsive to hedgehog signaling (in cells that express GATA4/5/6) or extremely responsive to hedgehog signaling (in cells that express NKX3.2 and thus do not express GATA4/5/6). Because *Nkx3.2* expression in paraxial mesoderm requires SHH signaling specifically for its induction and BMP signaling for its maintenance (Murtaugh et al., 2001), it is possible that GATA factors only act to repress the initiation of *Nkx3.2* expression (in response to hedgehog signals) but not its maintenance (by BMP signals). Indeed, immature chondrocytes in the growth plate are known to express NKX3.2 (Provot et al., 2006) and GATA6 (Alexandrovich et al., 2008).

It is not yet clear whether NKX3.2 represses GATA gene induction by directly binding to the regulatory regions of these genes or by repressing the expression of an activator(s) of this gene family. In addition, it is unclear whether GATA factors directly or indirectly repress the induction of *Nkx3.2*, in the latter case by repressing the transcriptional activity of Gli transcription factors. Interestingly, NKX3.2 (Kim and Lassar, 2003) and GATA transcription factors (Benchabane and Wrana, 2003; Brown et al., 2004) have both been demonstrated to bind to BMP-regulated SMAD1/4. Thus, it is formally possible that NKX3.2 and GATA could compete for SMAD1/4 interaction and thereby block one another's activity. However, because a mutant form of NKX3.2 (Nkx3.2-NQ) that can bind to SMAD1/4 yet cannot bind DNA (Kim et al., 2003) failed to repress induction of *Gata4* expression by BMP signals, it seems unlikely that NKX3.2 represses GATA induction by merely competing for SMAD1/4 interaction.

Because GATA factors can block SHH-mediated induction of *Sox9*, our findings suggest that NKX3.2 promotes chondrogenic competence in the sclerotome in part by blocking the expression of GATA genes. Interestingly, we have found that GATA transcription factors can also repress the ability of Wnt and SHH signals to induce expression of the pro-myogenic transcription factor genes *Pax3*, *Myf5* and *Myod* in explants of PSM (supplementary material Fig. S5A). Conversely, PAX3 can block BMP-mediated induction of *Gata4/5/6* in PSM explants (supplementary material Fig. S5B). Thus, PAX3 might play an analogous role to NKX3.2 in the dermomyotome and maintain the responsiveness of this tissue to SHH signals by blocking the induction of GATA transcription factors.

The ability of NKX3.2 to block induction of lateral plate markers in paraxial mesoderm treated with BMP4 is reminiscent of the function of FOXC1 and FOXC2 in paraxial mesoderm, where these transcription factors have been shown to block the expression of intermediate mesoderm markers in this tissue (Wilm et al., 2004). Interestingly, NKX3.2 and GATA family members are also expressed in mutually exclusive patterns in the gut, with NKX3.2 expression being restricted to the gizzard musculature and GATA family members expressed in the adjacent endoderm. Such mutually exclusive expression patterns of NKX3.2 and GATA factors in adjacent tissues might allow these tissues (in which BMP and SHH signaling are known to be crucial) to display markedly different sensitivities to hedgehog signaling.

GATA4/5/6 repress hedgehog-responsive genes

McMahon and colleagues observed that the BMP antagonist noggin synergizes with SHH to induce sclerotomal gene expression *in vivo* and *in vitro* (McMahon et al., 1998), suggesting that BMP signals somehow repress hedgehog-dependent gene expression. In accordance with this finding, Stafford and colleagues demonstrated that the BMP antagonists noggin and gremlin are necessary to carve out a BMP signaling-free zone in the paraxial mesoderm, such that SHH signals from the notochord can induce the expression of sclerotomal markers and subsequent vertebral chondrogenesis (Stafford et al., 2011). Consistent with this prior work, we have observed that treatment of chicken paraxial mesoderm explants with BMP4 induces the expression of *Gata4/5/6* and concomitantly decreases the expression of hedgehog-responsive genes in response to SHH administration. Interestingly, expression of ectopic GATA4 or GATA5 can mimic the effects of BMP administration to paraxial mesoderm and similarly block the induction of hedgehog-responsive genes. These findings suggest that BMP signals attenuate hedgehog signaling in paraxial mesoderm in part by inducing GATA factor expression. The expression of various hedgehog-responsive genes in paraxial mesoderm displayed differential sensitivity to GATA

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inhibition. Induction of *Sox9* and *Nkx3.2* was strongly blocked by ectopic expression of GATA5 in PSM explants cultured in low or high levels of SHH. By contrast, induction of *Gli1*, patched 1 and patched 2 was most effectively blocked by ectopic expression of GATA5 in PSM explants cultured in relatively low levels of SHH. It is not clear whether this differential sensitivity to GATA factor expression reflects differing affinities of the regulatory regions of these various hedgehog targets for Gli transcription factors, GATA transcription factors, or both.

We found that GATA factors can interact with Gli proteins (as assayed by mammalian two-hybrid analysis), suggesting that GATA factors could potentially repress hedgehog target genes by piggy-backing on Gli proteins that directly bind to hedgehog-inducible transcriptional targets. Consistent with these findings, ChIP analysis in *Sufu*-deficient cells programmed to express GATA4 or GATA5 revealed that GATA factors bind to chromatin encompassing GATA or Gli binding sites that lie proximal to the *Gli1* TSS. Thus, GATA proteins are recruited to sites upstream of the *Gli1* gene by both protein:DNA and protein:protein interaction. Indeed, a GATA6 DNA-binding domain mutant that is unable to bind directly to DNA (at GATA binding sites) could nonetheless efficiently repress induction of ~3.6 kb Gli1-luciferase by co-transfected GLI1. Both GATA factors (Benchabane and Wrana, 2003; Brown et al., 2004) and C-terminally truncated GLI3 (i.e. Gli3R) (Liu et al., 1998) have been demonstrated to associate with SMAD1/4, raising the possibility that protein:protein interaction between Gli proteins and GATA transcription factors might be mediated by an intermediate bridging protein, such as SMAD1/4. We found that GATA factors recruit the transcriptional co-factor FOG1 to both GATA and Gli binding sites located in the *Gli1* regulatory region. Moreover, shRNA-mediated knockdown of FOG1 reversed GATA-mediated repression of *Gli1* expression in *Sufu*$^{-/-}$ cells. Thus, the absence or presence of FOG co-factors in a given tissue may regulate whether GATA factors repress hedgehog signaling *in vivo*.

Consistent with our finding that GATA factors attenuate hedgehog signaling *in vitro*, we observed that conditional loss of GATA6 in limb buds leads to a dramatic induction of hedgehog transcriptional targets in the anterior mesenchyme of the hindlimb bud, resulting in hindlimb polydactyly (Kozhemyakina et al., 2014). In the anterior mesenchyme of the developing hindlimb bud, GATA6 directly represses the expression of hedgehog target genes such as *Gli1* (by binding to its regulatory region) and *Shh* itself, in the latter case by binding to the enhancer that drives *Shh* expression specifically in the limb bud (Kozhemyakina et al., 2014). Silencing of GATA4/5/6 expression by genetic or epigenetic means has been implicated in the development of ovarian, lung and gastric cancers (reviewed by Zheng and Blobel, 2010). In light of our findings that these same GATA factors can repress the expression of hedgehog target genes, it will be interesting to evaluate whether cancers that have lost GATA family member expression display increased expression of hedgehog target genes.

MATERIALS AND METHODS

Plasmids and viruses

Mouse *Gata4/5/6* cDNAs were provided by Dr Eric Olson (UT Southwestern). pCAGGS-GATA4, pCAGGS-GATA5, pCAGGS-GATA4-ZF and GATA4-ZF-VP16 are described by Kamei et al. (2011). pJT4-Shh, pCMV-Gal4-hGli1, pCMV-Gal4-hGli3, 8×3′-Gli-BS-Luc and 8×3′-mutant Gli-BS-Luc were provided by Dr Hiroshi Sasaki (Osaka University). pCFP-N3 mSmo-M2 was provided by Dr Adrian Salic (Harvard Medical School). ~3.6 kb Gli1-firefly luciferase (Dai et al., 1999) was provided by Dr Shunsuke Ishii (RIKEN, Japan). pcDNA3-GATA6-HA and pcDNA3-GATA6-DBDmut (which contains a mutation to Gly of the third Cys of the C-terminal zinc-finger) are described by Kozhemyakina et al. (2014).

Explant cultures, RCAS infection and gene expression analysis

Dissection and culture of PSM explants were performed as described (Zeng et al., 2002; Cairns et al., 2008). Reverse transcription and PCR analyses of explants were carried out as described previously (Zeng et al., 2002; Cairns et al., 2008).

Cell culture, transient transfection and luciferase assays

NIH 3T3 cells were maintained in DMEM supplemented with 10% FBS and 1× penicillin/streptomycin. FuGENE 6 (Promega) was used for all transfection assays as per manufacturer's instructions. Cells were 60-70% confluent and were transfected in the presence of 10% FBS. Twenty-four hours after transfection, cells were 100% confluent and medium was changed to DMEM supplemented with ITS (insulin, transferrin and selenium) for another 24 h. Thereafter, cells were washed once with PBS, lysed in passive lysis buffer and luciferase measured using a Dual Luciferase Assay Kit (Promega) as per manufacturer's instructions.

Generation of GATA4- or GATA5-expressing *Sufu*$^{-/-}$ MEFs

Sufu$^{-/-}$ MEFs were infected with retroviruses encoding pOZ-FlagHA-puro (Kumar et al., 2009), pOZ-GATA4-FlagHA-puro or pOZ-GATA5-FlagHA-puro. Stable polyclones were selected using puromycin.

Electrophoretic mobility shift assay (EMSA) analysis of Gli and GATA binding

COS-7 cells were transfected with either pCAGGS-GATA4, pCAGGS-GATA5, pcDNA1-GATA6 or pcDNA3.1-Gli1 and nuclear extracts were prepared using a Nuclear Extract Preparation Kit (Active Motif) as per manufacturer's instructions. The probes used for Gli-BS and GATA-BS were described previously (Sasaki et al., 1997; Lee et al., 2004).

Western blot analyses

Western blots were performed using rabbit anti-GLI1 (V812; Cell Signaling, 2534; 1:1000), rabbit anti-FOG1 (ZFPM1 antibody; Genway Biotech, GWB-185378; 1:1000) and mouse anti-HA (Santa Cruz Biotechnology, SC-805; clone Y-11 at 1:1000).

Chromatin immunoprecipitation (ChIP)

Sufu$^{-/-}$ MEFs that were infected with either pOZ-FlagHA-puro (the parental retrovirus) (Kumar et al., 2009), pOZ-GATA4-FlagHA-puro or pOZ-GATA5-FlagHA-puro were employed for ChIP as described previously (Carroll et al., 2005). Rabbit anti-FOG1 (as above), rabbit anti-HA (Santa Cruz Biotechnology, as above) or control rabbit IgG (Jackson Lab; 1:1000) was employed for ChIP. Primers amplified the following regions of the murine *Gli1* gene: P1, +1157 to +1312 (product 155 bp); P2, −1077 to −838 (239 bp); P3, −1572 to −1364 (208 bp); P4, −2309 to −2062 (247 bp); P5, −6799 to −6499 (300 bp); F1, +1004 to +1089 (85 bp); F2, +466 to +639 (173 bp); F3, +215 to +388 (173 bp); F4, −75 to −198 (124 bp). The statistical significance of ChIP results with various antibodies was calculated using Student's *t*-test.

shRNA-mediated knockdown of FOG1

Lentiviral vectors (pLKO.1-Puro) expressing FOG1shRNA or GFPshRNA were obtained from Open Biosystems. The mature sense sequence of the FOG1shRNA was CGAGATCACCTTTAACAATAT. *Sufu*$^{-/-}$ cells that were previously selected to express GATA4 or GATA5 were infected with retrovirus encoding FOG1shRNA or GFPshRNA.

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Competing interests

The authors declare no competing financial interests.

Author contributions

G.D., H.K. and A.B.L. were responsible for the conception and design of the study, analysis and interpretation of data, and drafting the article. G.D., H.K., D.K., E.K., T.H., D.-W.K. and A.I. were responsible for experimental design and data acquisition.

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Supplementary material

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