

## RESEARCH ARTICLE

# A Gli silencer is required for robust repression of gremlin in the vertebrate limb bud

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**ABSTRACT**

The transcriptional response to the Hedgehog (Hh) pathway is mediated by Gli proteins, which function as context-dependent transcriptional activators or repressors. However, the mechanism by which Gli proteins regulate their target genes is poorly understood. Here, we have performed the first genetic characterization of a Gli-dependent cis-regulatory module (CRM), focusing on its regulation of *Grem1* in the mouse limb bud. The CRM, termed GRE1 (Gli responsive element 1), can act as both an enhancer and a silencer. The enhancer activity requires sustained Hh signaling. As a Gli-dependent silencer, GRE1 prevents ectopic transcription of *Grem1* driven through additional CRMs. In doing so, GRE1 works with additional GREs to robustly regulate *Grem1*. We suggest that multiple Gli CRMs may be a general mechanism for mediating a robust transcriptional response to the Hh pathway.

**KEY WORDS:** Enhancer, Gli, Gremlin, Limb development, Silencer, Sonic Hedgehog, Mouse

**INTRODUCTION**

The Hedgehog (Hh) pathway is one of the primary signaling mechanisms underlying developmental patterning in organisms ranging from *Drosophila* to mammals (Ingham et al., 2011). Although progress has been made in understanding the processes underlying signal transduction and the genetic consequences of perturbing Hh signaling, the transcriptional mechanism by which Hh signaling regulates expression of its target genes remains poorly understood.

In vertebrates, Hh ligands ultimately regulate transcription by controlling the activity of Gli transcription factors (Gli1-3), homologs of the *Drosophila* Cubitus Interruptus (Ci) protein. In the presence of Hh signaling, full-length, activated Gli proteins translocate into the nucleus where they activate transcription (Bai et al., 2004; Bowers et al., 2012; Dai et al., 1999; Matise et al., 1998; Sasaki et al., 1999; Wang et al., 2000, 2007). In the absence of Hh signaling, Gli2 and Gli3 are processed by proteolytic cleavage into truncated proteins that act as transcriptional repressors (Litingtung et al., 2002; Pan et al., 2006; Wang et al., 2000; Wen et al., 2010). Evidence from a number of studies indicates that all forms of Gli transcription factors can bind the same 9 bp motif sequence (Hallikas et al., 2006; Muller and Basler, 2000; Peterson et al., 2012). The transcriptional output of Gli target genes is influenced both by the quality of the Gli motif sequence and the presence of tissue-specific co-factors. In addition, the genomic regions surrounding Gli target genes often contain multiple Gli-

binding regions, suggesting the possibility that multiple cis-regulatory modules (CRMs) could interact together to regulate gene expression (Biehs et al., 2010; Oosterveen et al., 2012; Peterson et al., 2012; Vokes et al., 2008). If these interactions exist, they could potentially result in redundant, synergistic or dose-dependent regulation of target genes.

Gli transcriptional targets fall into two distinct groups: genes that require Gli activation for transcription (Gli activator genes), and genes that are transcribed in the absence of Gli repression (Gli derepression genes). Gli activators could potentially play quantitative roles in regulating the expression levels of a subset of this latter class. The behavior of target genes in response to a gradient of Hh signaling suggests that competition between Gli activators and repressors could drive threshold responses that restrict the boundary of Gli-activator target gene expression (Jacob and Briscoe, 2003; Ruiz i Altaba, 1997; Wang et al., 2000). Studies that have manipulated Gli expression levels in the chick neural tube support this competition model (Oosterveen et al., 2012). The mechanism by which Gli repression prevents expression of its target genes is poorly understood, but in some cases relies on interactions between Gli repressors and specific transcription factors (Oosterveen et al., 2012). Mouse neural tubes lacking the major Gli transcriptional repressor Gli3 have a relatively modest change in target gene expression boundaries with no change in ventral neural fates and more subtle changes to intermediate identities, an effect that could be due to the robustness of the neural-specific downstream regulatory network (Balaskas et al., 2012; Persson et al., 2002).

Sonic hedgehog (Shh), the Hh ligand expressed in the limb bud, has graded activity emanating from the most posterior region of the limb bud. The dose and duration of Shh signaling are crucial for specifying digits and regulating growth (Ahn and Joyner, 2004; Harfe et al., 2004; Towers et al., 2008; Yang et al., 1997; Zhu et al., 2008). Compared with *Shh*<sup>-/-</sup> embryos, *Shh*<sup>-/-</sup>;*Gli3*<sup>-/-</sup> embryos have a substantial rescue in limb growth and digit formation. The expression of many genes that are lost in *Shh*<sup>-/-</sup> limb buds are restored in *Shh*<sup>-/-</sup>;*Gli3*<sup>-/-</sup> embryos but with symmetrical gene expression patterns along the anterior-posterior axis. This contrasts with their asymmetric expression in wild-type embryos and is exemplified by *Grem1*, an important Shh target gene that encodes a protein playing key roles in regulating differentiation (reviewed by Rabinowitz and Vokes, 2012). Expression of *Grem1* in the limb expands anteriorly in *Gli3*<sup>-/-</sup> embryos, is severely downregulated in *Shh*<sup>-/-</sup> embryos and is rescued in *Shh*<sup>-/-</sup>;*Gli3*<sup>-/-</sup> embryos (Aoto et al., 2002; Litingtung et al., 2002; Panman et al., 2006; te Welscher et al., 2002; Zúñiga et al., 1999). Collectively, these studies illustrate the profound importance of Shh in counteracting Gli3-mediated repression of target genes.

Despite the central role of Gli proteins in regulating Hh signaling responses, the mechanism by which Gli activator and repressor proteins collaboratively regulate target genes remains poorly understood and is an impediment to defining target genes and gene regulatory networks. We used a CRM that is embedded within a global control region for the mouse gremlin locus to perform the first genetic characterization (loss of function) of a Gli-responsive CRM.

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We find that the GRE1 (Gli responsive element 1) acts as both an enhancer and a silencer. GRE1 enhancer activity requires sustained Hh signaling to drive activity. In the anterior limb bud, GRE1 acts as a Gli-dependent silencer. The silencer activity is necessary for providing robust repression of *Grem1* in the distal-anterior limb.

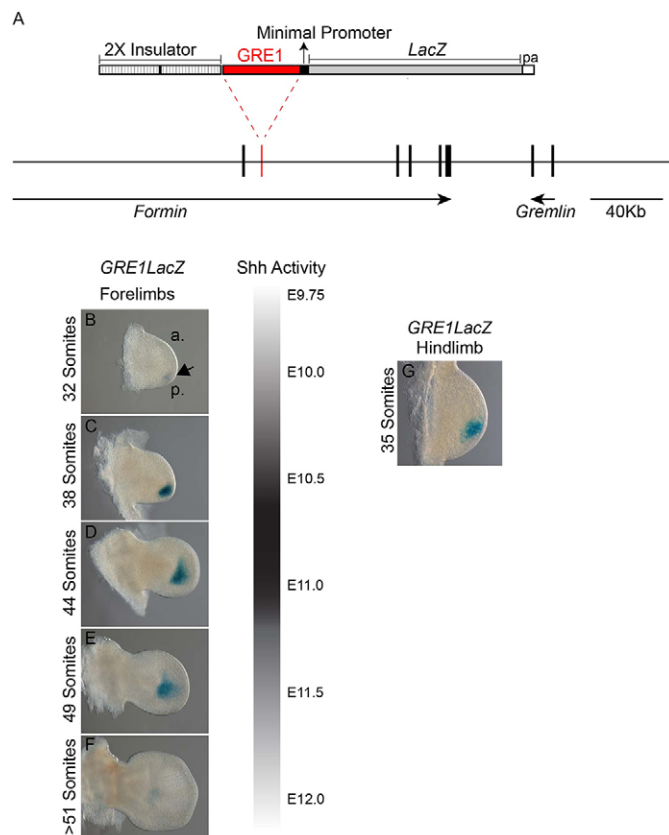
## RESULTS

In a genome-wide chromatin immunoprecipitation study, we previously identified a 438-bp Gli3-binding region located >100 kb downstream of *Grem1* that exhibited enhancer activity in transient transgenic limb buds in a region partially overlapping with *Grem1* gene expression (Fig. 1A) (Vokes et al., 2008). Enhancer activity is dependent on the presence of at least one Gli motif as mutations of the motif resulted in a complete lack of enhancer activity in G0 transgenic embryos (Vokes et al., 2008). We sought to characterize Gli enhancer regulation in the context of this CRM, which is henceforth referred to as GRE1 (Gli responsive element 1). Embryos derived from three founder lines of stable transgenics had  $\beta$ -galactosidase activity in posterior limb bud mesenchyme in an identical domain to that previously reported for transient transgenics (Vokes et al., 2008). We selected one line, Tg(Rr26-lacZ)438Svok, henceforth referred to as *GRE1LacZ*, for further analysis.  $\beta$ -Galactosidase activity was first detected in embryos at embryonic day (E) 10.0 (31-32 somites; Fig. 1B), well after the reported onset of *Grem1* expression at ~E9

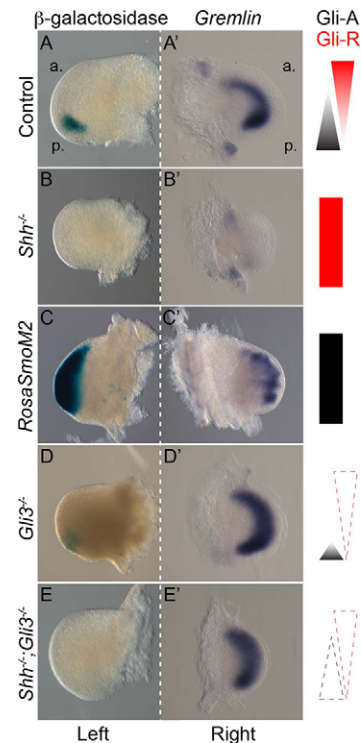
(Benazet et al., 2009; Zúñiga and Zeller, 1999). The enhancer had activity in the posterior limb within a subregion of the Shh-responsive domain. *Shh* expression initiates in the limb bud at ~28 somites (E9.75) (Charité et al., 2000), and the lag in reporter expression is consistent with the reported kinetics of Shh-mediated induction of *Grem1* (Benazet et al., 2009). By E10.5,  $\beta$ -galactosidase activity was strongly upregulated and persisted until late E11.5. By E11.75, expression was reduced and had retreated from the distal limb mesenchyme. Expression was nearly absent by E12.0 except for faint staining in the proximal middle of the condensing digit mesenchyme (Fig. 1C-F). No expression was detected after E12.0, correlating with the termination of Shh activity in the limb (Echelard et al., 1993; Harfe et al., 2004). Although the enhancer analyses focused on forelimb expression, we observed similar domains in the hindlimbs (Fig. 1G).

### Enhancer activity requires Shh signaling

To determine if GRE1 is responsive to Shh signaling, we examined enhancer activity at E10.5 in Shh gain- and loss-of-function backgrounds. In contrast to wild-type or heterozygous littermates, *Shh*<sup>-/-</sup>; *GRE1LacZ*<sup>+/-</sup> embryos had no detectable  $\beta$ -galactosidase activity (6/6 embryos) and, consistent with previous studies, *Grem1* gene expression was highly downregulated (Fig. 2B,B') (Zúñiga and Zeller, 1999). We also examined expression by activating high



**Fig. 1. GRE1 enhancer activity correlates temporally with Shh activity in mouse.** (A) Schematic showing the location of GRE1 in relation to the gremlin locus and the GRE1LacZ transgenic construct. (B-G) *GRE1LacZ*<sup>+/-</sup> forelimbs from E10 to E12 (B-F) and hindlimb from E10.5 (G) embryos stained for  $\beta$ -galactosidase activity. The somite stage is indicated to the left of each image. Expression activity in the limb correlates with *Shh* expression in the limb bud (indicated by the bar in the center). a., anterior; p., posterior.



**Fig. 2. GRE1 enhancer activity requires Gli activation.** (A-E') E10.5 *GRE1LacZ* forelimbs indicating enhancer activity in various genetic backgrounds. The corresponding Gli gradient status (Gli activator in black, Gli repressor in red) is indicated to the right of each set of images. Embryos were dissected into halves; the left forelimb (left column) was stained for enhancer activity ( $\beta$ -galactosidase) and the corresponding right forelimb (right column) was assayed for *Grem1* gene expression. Limbs on the left appear larger than their contralateral right side as a result of differences in how they are fixed. The genotypes at the left correspond to *GRE1LacZ*<sup>+/-</sup> (control; A,A'), *Shh*<sup>-/-</sup>; *GRE1LacZ*<sup>+/-</sup> (B,B'), *GRE1LacZ*<sup>+/-</sup>, *PrxCre*<sup>+/-</sup>; *Rosa*<sup>SmoM2cl/+</sup> (C,C'), *Gli3*<sup>-/-</sup>; *GRE1LacZ*<sup>+/-</sup> (D,D') and *GRE1LacZ*<sup>+/-</sup>; *Shh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup> (E,E'). a., anterior; p., posterior.

levels of Hh signaling throughout the limb bud using a Cre-inducible, dominant-active allele, *Rosa<sup>SmoM2</sup>* (Jeong et al., 2004). *Prx1Cre;Rosa<sup>SmoM2c/+</sup>;GRE1LacZ<sup>+/-</sup>* embryos expressed both the *Grem1* transcript and  $\beta$ -galactosidase activity throughout the entire distal limb bud (11/11 embryos; Fig. 2C,C'), indicating that high levels of Hh pathway activity were sufficient to activate *GRE1LacZ* along the anterior-posterior axis. *Grem1* gene expression appeared patchy (Fig. 2C'), and although the reason for this expression is unclear, it is consistent with observations from another study that also activated the Hh pathway throughout the limb bud (Butterfield et al., 2009). Because *Prx1Cre* is active throughout the limb mesenchyme (Logan et al., 2002), the distal restriction of GRE1 enhancer activity suggested that additional, distal factors are also required for *Grem1* expression. We concluded that Shh is both necessary and sufficient for enhancer activation.

### The enhancer domain is regulated by Gli activation

We next examined enhancer activity in *Gli3<sup>-/-</sup>;GRE1LacZ<sup>+/-</sup>* embryos at E10.5. Consistent with previous reports, *Grem1* gene expression expands anteriorly in *Gli3<sup>-/-</sup>* embryos (Fig. 2D'). By contrast, the enhancer activity domain, marked by  $\beta$ -galactosidase staining, does not expand anteriorly (Fig. 2D). Instead, the domain is significantly reduced in all *Gli3<sup>-/-</sup>* embryos (5/5) compared with heterozygous littermates ( $P=0.0007$ ). The reduction in enhancer activity suggests a role for Gli3 as an activator in the posterior limb. Consistent with this, *Gli3<sup>-/-</sup>* limbs at this stage had significantly reduced levels of the Gli activator target gene *Gli1* (supplementary material Fig. S1A). There was also a trend towards a 25% reduction in Shh levels that did not reach statistically significant levels (supplementary material Fig. S1B). These results are consistent with previous studies that have shown that *Gli3<sup>-/-</sup>* limb buds have reduced Gli activator levels as a combination of the direct reduction in Gli3 activator and reduced levels of *Shh* (Bai et al., 2004; Galli et al., 2010; Wang et al., 2007).

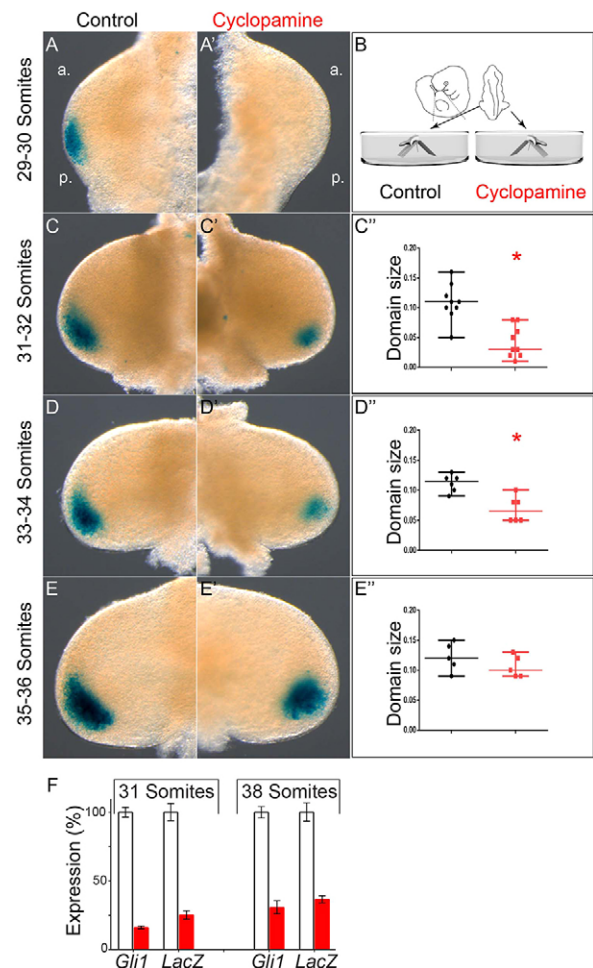
In *Shh<sup>-/-</sup>;Gli3<sup>-/-</sup>;GRE1LacZ<sup>+/-</sup>* embryos at E10.5, *Grem1* expression persists in limb buds in a depolarized fashion, as shown previously (Fig. 2E') (Aoto et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002). However, the limb buds had an absence of  $\beta$ -galactosidase staining (3/3 embryos; Fig. 2E), indicating that Gli activation is required for GRE1 enhancer activity. This is consistent with our previous work that identified a Gli motif that was essential for driving enhancer activity (Vokes et al., 2008). Although we focused on enhancer activity at E10.5, we noticed a single *Shh<sup>-/-</sup>;Gli3<sup>-/-</sup>;GRE1LacZ<sup>+/-</sup>* embryo at E11 that had a thin stripe of  $\beta$ -galactosidase activity extending in a symmetrical arc across the distal limb bud (supplementary material Fig. S2E). A similar arc was seen in the anterior of E11 *Gli3<sup>-/-</sup>;GRE1LacZ* embryos whereas no activity was present in *Shh<sup>-/-</sup>* embryos (supplementary material Fig. S2). It is presently unclear whether this expression reflects a weak, late role for Gli3 repression in restricting the enhancer domain or some type of indirect activation (see Discussion).

### Enhancer activity requires sustained Gli activation

To determine the time period during which GRE1 requires Gli activator for enhancer activity in the posterior limb, we used an established *ex vivo* limb bud culture assay, treating *GRE1LacZ<sup>+/-</sup>* limb buds with the Hh pathway inhibitor cyclopamine (Panman et al., 2006). We cultured one forelimb in media containing cyclopamine while the contralateral side was cultured in control media, providing an internal control for staging and embryo variability (Fig. 3B). As expected from the lack of activity in

*Shh<sup>-/-</sup>* embryos (Fig. 2B), limb buds cultured in cyclopamine at stages before enhancer activity is detected (29–30 somites) resulted in a complete loss of  $\beta$ -galactosidase (Fig. 3A,A'). In limb buds cultured at 31–32 somites, there is a strong reduction (61%) in the size of the enhancer activity domain compared with the control side (Fig. 3C-C';  $P=0.0004$ ). Limbs cultured at 33–34 somites have more modest reductions (39%) in the size of the enhancer domain (Fig. 3D-D';  $P=0.0065$ ). The domain size no longer depends on Shh signaling from 35–36 somites onwards (Fig. 3E-E';  $P=0.3333$ ). These results indicated that Shh signaling is required for expanding the domain of enhancer activity until 35–36 somites.

Because residual  $\beta$ -galactosidase protein could persist after the cessation of transcriptional activity from the reporter, it was not possible to determine if Shh is required to maintain enhancer activity



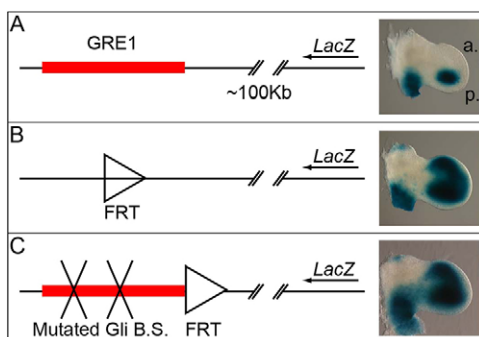
**Fig. 3. GRE1 enhancer activity requires sustained Shh signaling.** (A-E') *GRE1LacZ<sup>+/-</sup>* forelimbs were cultured in vehicle-containing control media (A-E) whereas their contralateral forelimbs were cultured in cyclopamine (A'-E'), as shown in the schematic (B). The stage of the limb buds at the start of the experiment is indicated on the left. (C''-E'') Graphs indicating the domain size measured by the ratio of the  $\beta$ -galactosidase-stained area to the total limb bud area for control (black) or cyclopamine-treated (red) limb buds. Data points indicate the median and range of values. The presence of an asterisk indicates statistically significant difference (Mann-Whitney U test). Specific values are: C'':  $U=3.5$ ,  $P=0.0004$ ; D'':  $U=1.5$ ,  $P=0.0065$ ; E'':  $U=7.5$ ,  $P=0.3333$ . (F) Quantitative RT-PCR experiments from single forelimbs (red bars) and their contralateral control limbs (white bars) for representative 31- and 38-somite embryos cultured in control and cyclopamine-containing media (error bars indicate the s.e.m.). a., anterior; p., posterior.

with this approach. To circumvent this problem, we performed additional limb bud cultures on 32- and 38-somite embryos and measured *lacZ* expression by qRT-PCR. As a control to ensure that the experimental conditions resulted in robust inhibition of Shh signaling, we measured the expression of the obligate Shh target gene *Gli1* (Panman et al., 2006). When forelimbs from 32-somite embryos were cultured, they had an 84% reduction in *Gli1* gene expression and a 75% reduction in *lacZ* expression. Similarly, forelimbs cultured from 38-somite embryos had a 70% reduction in *Gli1* and also had a 64% reduction in *lacZ* (Fig. 3F). The change in gene expression at later stages contrasts with the stable expression domains indicated by  $\beta$ -galactosidase staining (Fig. 3E–E’). We concluded that establishing the enhancer domain requires Shh signaling transiently until 35 somites, whereas enhancer activity within the domain continues to require sustained Shh signaling.

In addition to requiring Hh signaling, GRE1 could potentially be negatively regulated by fibroblast growth factor (FGF) signaling, which has previously been shown to negatively regulate *Grem1* during later limb specification (Verheyden and Sun, 2008). To test this, we cultured additional *GRE1lacZ* limb buds in the presence and absence of the FGF inhibitor SU5402 (Mohammadi et al., 1997). Consistent with previous results, *Grem1* was expanded in the distal-anterior limbs in SU5402-treated cultures, but *GRE1lacZ* activity was not inhibited by FGF signaling (supplementary material Fig. S3).

#### Gli repression of the CRM prevents ectopic anterior expression of *Grem1*

To determine the effect of GRE1 on *Grem1* expression, we examined G0 transgenics at E11–11.5 (41–48 somites) containing a previously generated bacterial artificial chromosome (BAC) in which *lacZ* was inserted into the *Grem1* coding region (Zuniga et al., 2004). Consistent with previous studies,  $\beta$ -galactosidase activity was restricted to the posterior limb in  $\beta$ -galactosidase-expressing embryos (0/6 have anterior expression; Fig. 4A) (Zuniga et al., 2012, 2004). The expression domain was similar although not identical to *GRE1lacZ* expression at the same stage (Fig. 1D), and we hypothesized that deletion of GRE1 would reduce or eliminate reporter gene expression. Unexpectedly, the deletion resulted in ectopic anterior limb bud expression in most embryos (7/10; Fig. 4B). The ectopic anterior expression suggested the presence of

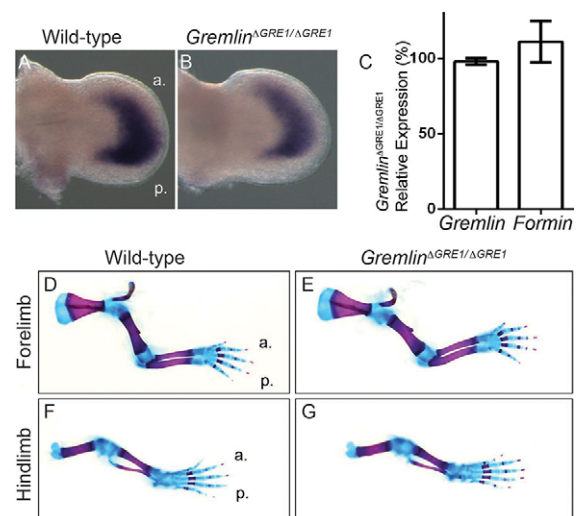


**Fig. 4. GRE1 is necessary for Gli repression of *Grem1* in the anterior limb.** (A–C) BAC constructs have *lacZ* inserted into the *Grem1* coding region to act as a reporter of its transcriptional expression. Representative forelimb buds are shown from E11 G0 transgenic embryos containing the indicated BAC construct. *Grem1* expression from the unaltered BAC transgene is present exclusively in the posterior limb bud (A). Deletion of GRE1 (B) or mutation of the two Gli motifs present within GRE1 (C) results in ectopic distal-anterior expression. a., anterior; p., posterior.

additional CRM(s) that either individually or collectively have pan-limb enhancer activity. It also suggested that GRE1 is acting as a silencer in the anterior limb. We generated a third set of constructs in which GRE1 was re-inserted into the BAC with mutations in the two Gli DNA-binding motifs present in the CRM (see Materials and Methods). These forelimbs also contained ectopic anterior expression (3/3 embryos; Fig. 4C). We concluded that Gli-binding regions within GRE1 mediate silencer activity, preventing ectopic *Grem1* transcription. This is consistent with the anterior expansion of *Grem1* observed in *Gli3*<sup>-/-</sup> embryos (Fig. 2D’).

#### The CRM functions as a Gli-mediated silencer

The results described so far suggested that GRE1 likely mediates the transcriptional repression of *Grem1*. We then generated mice containing a deletion of GRE1 (supplementary material Fig. S4). *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> forelimbs expressed gremlin and formin 1 at levels that are indistinguishable from wild-type control forelimbs (Fig. 5A–C). *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> mice were viable and fertile with normal skeletal patterning (Fig. 5D–G). Embryos containing one null allele of *Grem1* (Khokha et al., 2003) and a second allele harboring the deletion of GRE1 also had normal skeletal patterning (supplementary material Fig. S5). These results indicate that GRE1 is not necessary for normal skeletal development. The *Grem1* gene expression domain was nearly normal in *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> embryos at E11 (Fig. 5A,B), but at earlier stages, the distal anterior boundaries of expression were more diffuse (Fig. 6A,B). In light of these results, we hypothesized that redundant Gli-dependent CRMs might regulate *Grem1*. Two additional Gli-binding regions are present within the gremlin locus (Vokes et al., 2008). One of these regions was recently shown to have Shh-responsive enhancer activity and to be crucial for mediating BAC reporter activity in transgenic embryos (Zuniga et al., 2012). We hypothesized that our Gli CRM might be redundant with other GREs under normal conditions but still required for robust regulation of *Grem1*.



**Fig. 5. GRE1 is not essential for limb development.** (A,B) *Grem1* expression in forelimbs at E11 (41 somites). (C) *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> embryos express gremlin and formin 1 at levels that are statistically indistinguishable from wild-type embryos (one sample *t*-test, two tails; gremlin,  $P=0.1172$ ; formin 1,  $P=0.4548$ ). Values are normalized to the distal marker jagged 1. Error bars represent s.e.m. from four independent biological samples (E10.5, 34–37 somites). (D–G) E18.5 skeletal preparations showing forelimbs and hindlimbs of the indicated genotypes. a., anterior; p., posterior.

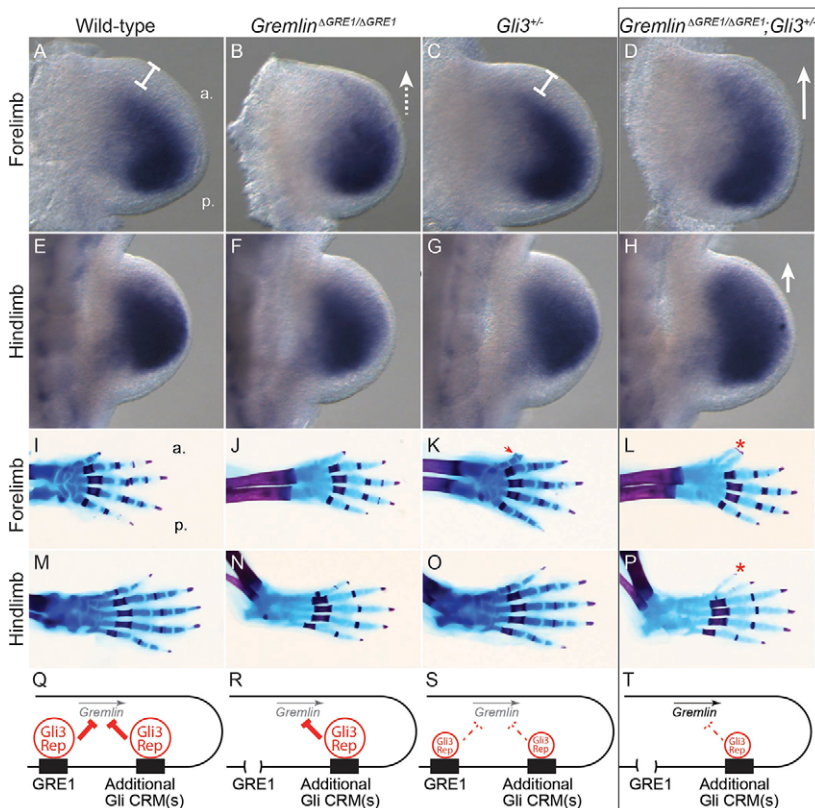
Studies in *Drosophila* have tested the robustness of transcriptional responses to shadow enhancers by examining CRM deletion phenotypes at the outer ranges of permissive temperatures or by removing one copy of an upstream regulator (Frankel et al., 2010; Perry et al., 2010). We used the latter strategy to examine *Gremlin* expression in *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> embryos containing a single copy of *Gli3*, which is sufficient to prevent the distal-anterior expression of *Greml* seen in *Gli3*<sup>-/-</sup> embryos (te Welscher et al., 2002) (Fig. 2D'). At E10.5, both wild-type and *Gli3*<sup>+/-</sup> littermates have a sharp boundary of *Greml* expression that is restricted from the most distal-anterior mesoderm in the forelimbs ( $n=7$ ; Fig. 6A,C, brackets). *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> littermates have forelimbs with less pronounced distal-anterior borders of *Greml* and with weak ectopic expression in the anterior limb mesoderm directly adjacent to the apical ectodermal ridge ( $n=10$ ; Fig. 6B, dashed arrow). By contrast, *Gremlin*<sup>ΔGRE1/ΔGRE1</sup>; *Gli3*<sup>+/-</sup> littermates have forelimbs with ectopic distal-anterior *Greml* expression that is broader and stronger than in *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> forelimbs ( $n=8$ ; Fig. 6D). This expression is significantly different from *Gli3*<sup>+/-</sup> ( $P=0.0002$ ) or *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> forelimbs ( $P<0.0001$ ), indicating a genetic interaction between *Gli3* and the *Gremlin*<sup>ΔGRE1</sup> allele.

An expansion of GREM1 into the anterior distal mesenchyme would inhibit bone morphogenetic proteins (BMPs), causing an expansion in anterior growth (Lopez-Rios et al., 2012; Pizette and Niswander, 1999). This growth would probably result in anterior polydactyly, which is also seen in mice with reduced BMP activity (Dunn et al., 1997; Selever et al., 2004). In the mixed genetic background present in our colony, the presence of the *Gli3*<sup>+/-</sup> 'extra toes' allele only rarely results in mice or embryos with fully polydactylous digits. In this study, all of the *Gli3*<sup>+/-</sup> embryos had a single nub (a fleshy outgrowth that sometimes contains a single speck of cartilage) but none of them had distinct polydactylous digits (18/18 hindlimbs; supplementary material Table S1; Fig. 6O).

*Gremlin*<sup>ΔGRE1/ΔGRE1</sup> littermates have normal digit patterning (14/14 hindlimbs; supplementary material Table S1; Fig. 6N). By contrast, *Gremlin*<sup>ΔGRE1/ΔGRE1</sup>; *Gli3*<sup>+/-</sup> littermates have a distinct, polydactylous digit in three out of eight hindlimbs (Fig. 6P), a significant difference from *Gli3*<sup>+/-</sup> embryos ( $P=0.0215$ ). *Gli3*<sup>+/-</sup> forelimbs displayed a spectrum of phenotypes ranging from completely normal digits (7/17) to polysyndactyly (4/17) (supplementary material Table S1). *Gremlin*<sup>ΔGRE1/ΔGRE1</sup>; *Gli3*<sup>+/-</sup> forelimbs uniformly contained a polysyndactylous thumb (8/8), a significant increase in frequency compared with *Gli3*<sup>+/-</sup> embryos ( $P=0.0005$ ; Fig. 6I-L). *Gremlin*<sup>ΔGRE1/+</sup>; *Gli3*<sup>+/-</sup> embryos also contained a high proportion of polysyndactylous forelimbs (23/28; supplementary material Table S1). These results suggest that GRE1 has silencer activity that is required for robust anterior repression of *Greml*. Our result is consistent with previous studies showing a genetic interaction between *Gli3* and BMP4 (Dunn et al., 1997; Lopez-Rios et al., 2012). To determine if GRE1 might also be required to provide a Gli activator input for robust Gli enhancer activity, we performed a parallel analysis of compound crosses with *Shh*<sup>+/-</sup> mice. *Gremlin*<sup>ΔGRE1/ΔGRE1</sup>; *Shh*<sup>+/-</sup> embryos have no genetic interaction (supplementary material Fig. S6), suggesting that the enhancer properties of GRE1 are either completely redundant or biologically irrelevant. We concluded that silencer activity through GRE1 is required for robust, Gli-dependent repression of *Greml* in the anterior limb (schematized in Fig. 6Q-T).

## DISCUSSION

In this study, we have performed the first genetic characterization of a vertebrate Gli CRM. Within the limb bud, most putative Gli target genes are associated with multiple Gli-binding regions (Vokes et al., 2008). Our results, summarized in supplementary material Table S2, suggest that one role for multiple, distinct Gli-binding regions around Gli target genes is to provide a robust silencing response that buffers against genetic perturbations. This is in contrast to the *Fgf8* and *HoxD*



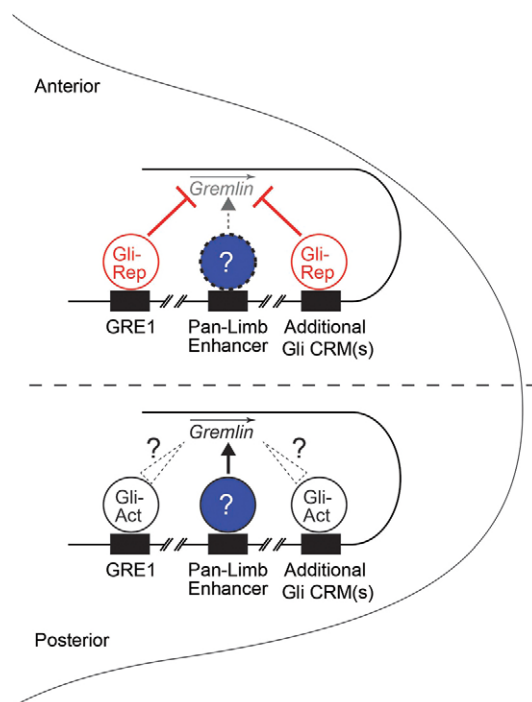
**Fig. 6. The GRE1 CRM interacts genetically with *Gli3* to repress *Greml*.** (A-H) *Greml* expression in various genetic backgrounds in the forelimbs of 35–36 somite embryos (A–D) and hindlimbs of 37–38 somite embryos (E–H). The white brackets in A and C indicate the *Greml*-free domain in anterior limb buds. White arrows (B,D,H) indicate ectopic distal-anterior *Greml* expression in *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> backgrounds. (I–P) E18.5 skeletal preparations of the hand (I–L) or foot (M–P) in various genetic backgrounds. The arrowhead (K) highlights a bifurcated thumb; the asterisks (L,P) indicate polydactylous digits. (Q–T) Schematic models showing how *Gli3* repression of *Greml* might occur in various genetic backgrounds.

loci, for which multiple enhancers with similar activity domains have been proposed to additively or synergistically amplify transcription (Marinić et al., 2013; Montavon et al., 2011). Our results further suggest that Gli silencers prevent transcriptional activity driven by additional, Gli-independent CRMs. We also show that GRE1 can act as a Gli-activator-dependent enhancer in the posterior limb, although the biological role for this activity is unclear (see section on Gli enhancer activity).

We propose a model in which Gli repressors bind to multiple Gli-dependent CRMs in the anterior limb, providing a robust silencing activity that prevents ectopic activation of *Grem1* that would otherwise be driven by at least one additional Gli-independent CRM that is active throughout the distal limb (a pan-limb enhancer). Gli repressor-mediated silencing results in the anterior repression of *Grem1* in the absence of threshold levels of Gli activator complexes. In the posterior limb, where Gli activator activity is high and Gli repressor activity is low, GRE1 silencing activity is lost and Gli-activator complexes provide enhancer activity. We have synthesized these results in a model for how *Grem1* is regulated by Gli proteins within the limb (Fig. 7).

### Gli enhancer activity

GRE1 enhancer activity is detected in the posterior limb in a spatial and temporal fashion that correlates with Shh signaling (Fig. 1). GRE1 requires Gli activation for initiating and sustaining activity at



### Fig. 7. Gli proteins generate asymmetric expression of gremlin.

*Grem1* is activated by a pan-limb enhancer (blue circle) that has activity throughout the distal limb. In genetic backgrounds in which there is an absence of Gli regulation (no activation or repression, e.g. *Shh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup>), the pan-limb enhancer drives symmetrical expression of *Grem1* throughout the limb bud. In the posterior region, Gli activators regulate redundant Gli-dependent CRMs, including GRE1, causing a loss of Gli-mediated silencing and possibly threshold-dependent enhancer activity (indicated by dashed triangles). In the anterior region, GRE1 acts as a silencer, preventing ectopic activation of *Grem1* through a pan-limb enhancer. The additional Gli-dependent CRM(s) could be either directly or indirectly regulated by Gli signaling.

E10.5 and ectopic Gli activator signaling is sufficient to drive GRE1 expression throughout the anterior-posterior axis (Fig. 2). These results suggest that GRE1 enhancer activity is primarily regulated by Shh signaling. GRE1 enhancer activity is transiently reduced in E10.5 *Gli3*<sup>-/-</sup> limb buds (Fig. 2D). Our results (supplementary material Fig. S1) are consistent with several studies showing that *Gli3*<sup>-/-</sup> limbs have reduced levels of Gli activation caused by a combination of reduced levels of Gli proteins and a reduction in *Shh* (Bai et al., 2004; Bowers et al., 2012; Galli et al., 2010; Wang et al., 2007).

In marked contrast to *Grem1* gene expression, the GRE1 enhancer domain does not expand in E10.5 *Gli3*<sup>-/-</sup> limb buds (Fig. 2D,D'). We rule out the possibility that Gli repressors do not work through GRE1 because our subsequent experiments indicate that it does indeed mediate Gli repressor-mediated silencing of *Grem1* (Figs 4, 6) and it is bound by Gli3 repressor in chromatin immunoprecipitation assays (Vokes et al., 2008). The behavior of GRE1 contrasts with the behavior of a *dpp* wing imaginal disc CRM in *Drosophila*, in which both repressor and activator functions of Ci can be detected in the same enhancer element (Muller and Basler, 2000). Within the mammalian neural tube, studies have reported conflicting conclusions regarding the role for Gli3 in restricting the boundaries of Gli activator enhancers or genes (Balaskas et al., 2012; Oosterveen et al., 2012; Peterson et al., 2012).

In contrast to the absence of anterior expression at E10.5, there is a thin, anterior domain of enhancer activity at E11 in *Gli3*<sup>-/-</sup> and *Shh*<sup>-/-</sup>; *Gli3*<sup>-/-</sup> embryos (supplementary material Fig. S2C-E). Previous experiments with G0 transgenics indicated that a Gli motif within GRE1 is absolutely required for enhancer activity at E11 (Vokes et al., 2008). It is presently unclear whether this expression reflects a weak, late role for Gli3 repression in restricting the enhancer domain, an artifact of the enhancer construct or transgenic line, or some type of indirect activation. If this represents biological derepression, a possible model would be the presence of an unknown anterior activator in the anterior limb that is repressed by Gli3 but activates late GRE1 activity. This would be consistent with our previous G0 transgenic enhancer results because they were in wild-type limbs and so the hypothetical anterior activator would still be repressed (Vokes et al., 2008).

There are several possible explanations for the lack of anterior expansion of *GRE1LacZ* in E10.5 *Gli3*<sup>-/-</sup> limb buds. The first is that Gli repressors might not compete with Gli activators to limit the anterior domain of enhancer activity. In this scenario, enhancer activity is driven solely by threshold-dependent Gli activation. The lack of baseline anterior activity would prevent visualization of the silencer activity in an enhancer reporter assay. A second possibility is that the *GRE1LacZ* transgenic construct is incapable of responding normally to Gli repressors because it is removed from its normal chromosomal environment. Indeed, our experiments suggest that Gli repressors do regulate the activity of additional CRMs in the gremlin locus (Figs 4, 6). Taken out of context, GRE1 could also have altered affinities for Gli activator and repressor complexes that prevent its anterior expansion in *Gli3*<sup>-/-</sup> embryos. A third possibility is that residual Gli repressor activity is sufficient to prevent anterior expansion of *GRE1LacZ* in *Gli3*<sup>-/-</sup> limb buds. Consistent with this, recent work has indicated that there is a genetic role for Gli2 repressor in skeletal patterning in the absence of Gli3 (Bowers et al., 2012). However, *Grem1* expression appears largely symmetrical along the anterior-posterior axis in *Gli3*<sup>-/-</sup> limb buds, suggesting that the remaining Gli repressor activity mediated by Gli2 might not be sufficient to repress *Grem1* (Fig. 2D') (Aoto et al., 2002; Litingtung et al., 2002; te Welscher et al., 2002). Additional

studies examining *GRE1lacZ* enhancer activity in *Gli2<sup>-/-</sup>;Gli3<sup>-/-</sup>* limb buds would be necessary to determine if GRE1 itself is more sensitive to Gli2 repression than the overall *Grem1* gene expression pattern would suggest.

### Gli repressor activity

Two models for Gli repression have been proposed (Wang et al., 2010). In one, Gli3 repressor acts as an inert decoy competing with Gli activator to regulate the transcription of target genes (Oosterveen et al., 2012; Wang et al., 2010). In the second, Gli repressor behaves like a conventional transcriptional repressor, recruiting transcriptional co-repressors that actively shut down transcription (Wang et al., 2010). Whereas the first model would apply specifically to Gli activator target genes, the second model could, in principle, apply both to Gli activator target genes and to genes that only require Gli derepression. GRE1 displays properties that are associated with both classes of Gli target genes. *Grem1* is a Gli derepression gene and Gli3 works through GRE1 as a silencer, preventing transcription directed by additional CRMs that would otherwise lead to ectopic distal-anterior expression. This mechanism of repression is distinct from that of conventional CRMs, for which repressor activity is integrated at the CRM level with each CRM then acting as an autonomous module to regulate gene expression. In future studies, it will be interesting to determine the mechanism of repression, which could function as a basal regulator of transcriptional activity. Alternatively, Gli3 might specifically inactivate one or more CRMs.

### Gli proteins generate asymmetric gene expression

In the posterior limb bud, it is unclear whether Gli activators are simply indicative of a derepressed environment that permits additional CRMs to drive expression or if they also provide a quantitative contribution as enhancers to increase *Grem1* transcription. The only evidence suggesting GRE1 is an enhancer is the enhancer activity of the isolated element in transgenic limb buds (Figs 2, 3). Although this fits the generally accepted criteria for an enhancer, there is no genetic evidence for reduced Gli activator responses in either *Gremlin<sup>ΔGRE1/-</sup>* or *Gremlin<sup>ΔGRE1/ΔGRE1</sup>;Shh<sup>+/-</sup>* embryos (supplementary material Figs S5, S6). There is also no observable reduction of posterior β-galactosidase activity in the BAC transgenics harboring a deletion in GRE1 (Fig. 4B). The lack of any detectable phenotype suggests that in the context of the native genomic locus, the enhancer activity is absent, trivial or completely redundant with additional Gli-dependent CRMs. The ambiguity over the contribution of enhancer activity is represented in Fig. 7, suggesting that the major purpose of GRE1 enhancer activity lies in counteracting Gli repression rather than providing quantitative levels of activation. In this way, GRE1 could act as a binary switch, causing transcription to be on or off in different domains (Fig. 7). This model provides a mechanism for how Shh signaling imposes asymmetric expression of ‘pre-patterned’ genes that would, in the absence of any Gli regulation, be symmetrically expressed throughout the limb bud. It also suggests that the inclusion of Gli-driven CRMs into the locus of pre-patterned limb might have provided an evolutionary mechanism for regulating asymmetric gene expression in a pre-existing pattern.

### Multiple CRMs regulate gremlin

Within the context of this study, there appear to be at least three distinct CRMs regulating *Grem1*. This is consistent with previous studies that describe a complex regulatory locus for *Grem1* (Vokes et al., 2008; Wang et al., 1997; Zuniga et al., 2012, 2004). Several proteins have also been shown to regulate *Grem1* at various

developmental time points. In particular, BMPs and HoxA/D transcription factors both regulate *Grem1* along the anterior-posterior axis. Their activity and expression domains make them excellent candidate regulators for the Gli-independent pan-limb enhancer (Fig. 7) (Benazet et al., 2009; Capdevila et al., 1999; Nissim et al., 2006; Sheth et al., 2013). Intriguingly, HoxA/D conditional mutants lack most *Grem1* expression with the exception of a posterior domain that appears to be nearly identical to the Gli CRM enhancer domain (Fig. 1D) (Sheth et al., 2013). Although our model depicts pan-limb enhancer activity with one CRM as the simplest possibility (Fig. 7), it is certainly possible that this activity integrates multiple Gli-independent enhancers active in distinct or overlapping domains.

Recently, a second GRE that lies closer to the transcriptional start site has been characterized. Although it does not contain a high affinity Gli motif within the core region, it is nonetheless bound by Gli3 in chromatin immunoprecipitation assays and requires Shh expression for enhancer activity in mutant embryos (Zuniga et al., 2012). Unlike GRE1, the more proximal GRE is essential for *Grem1* transcriptional activity in the same BAC reporter used in this study (Zuniga et al., 2012). Notably, GRE1 is not sufficient to activate transcriptional activity in its absence. This more proximal GRE could integrate Gli signaling with additional, Shh-independent facets of *Grem1* or there could be additional, uncharacterized Gli-dependent element(s). Our study was limited to the contribution of a single CRM, and future studies will be required to determine if there are higher-order chromatin interactions among the individual CRMs regulating *Grem1*, as has been suggested for the *Fgf8* and *HoxD* loci (Marinić et al., 2013; Montavon et al., 2011). In *Drosophila*, Ci (Gli) repressors have been proposed to work cooperatively by binding to several distinct sites within a CRM regulating *dpp* (Parker et al., 2011). The presence of an additional Gli CRM in the gremlin locus raises the intriguing possibility that Gli proteins binding to distinct CRMs might nonetheless be able to cooperatively repress *Grem1* in the context of a higher order chromatin structure.

### Redundant Gli inputs as a mechanism for fostering robust transcriptional control

Given the crucial role for Shh in regulating *Grem1* and the significant derepression observed when GRE1 was deleted in transgenic BAC reporters (Fig. 4B), we were initially surprised at the subtle phenotypes seen upon deleting the Gli CRM. Embryos and mice lacking GRE1 have no detectable skeletal phenotype. Nonetheless, embryos do have subtle shifts in *Grem1* expression (Fig. 6A,B), and when one copy of *Gli3* is removed GRE1 is required for the repression of *Grem1*. It is possible that the enhanced phenotype seen in  $\Delta$ GRE1;*Gli3* compound heterozygous embryos (Fig. 6) is due to the presence of another allele co-segregating with GRE1. The primary support that this interaction occurs between GRE1 and *Gli3* is that it is consistent with interactions observed between Gli3 and BMPs (which should have reduced anterior activity with ectopic *Grem1* expression) (Dunn et al., 1997; Lopez-Rios et al., 2012).

Both the subtle changes in expression pattern and the requirement of the CRM as a mechanism for buffering genetic variation are analogous to the shadow enhancers described in *Drosophila* (Barolo, 2012; Frankel et al., 2010). Shadow elements are defined by the genetic interactions of two genetically defined CRMs (Frankel, 2012) and further genetic studies involving multiple Gli-bound elements would be required to determine if the Gli CRM is functioning as a shadow repressor of *Grem1*. Our study, focused

exclusively on a single Gli CRM, is the first to address the potential genetic role that multiple Gli-bound CRMs play in regulating transcription. Multiple Gli-binding sites are associated with many predicted Gli target genes (Peterson et al., 2012; Vokes et al., 2008) and we propose that they may act as a general mechanism for mediating robust transcriptional responses to Hh signaling.

## MATERIALS AND METHODS

### Ethics statement

Experiments involving mice were approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin (protocol AUP-2010-00166).

### Generation of mouse strains

The genomic coordinates in this study are reported in the Genome Reference Consortium mm10 build 38 genomic assembly. The *GRE1lacZ* transgenic line, officially named Tg(Rr26-lacZ)438Svok (MGI:5052053), was generated by pronuclear injection using the previously described enhancer reporter construct containing the 438-bp Gli binding region (chr2:113640843-113641280) (Vokes et al., 2008). The BAC transgenic constructs were generated using the Quick&Easy BAC Modification Kit (Gene Bridges) to modify a previously generated BAC containing *lacZ* within the *Grem1* transcript (Zuniga et al., 2004). The homology arms for both targeting vectors were chr2:113,640,295-113,640,842 and chr2:113,641,281-113,641,757. After targeting, the FRT-flanked neomycin-resistance cassette was removed with a heat shock-inducible FIpE construct (Gene Bridges), leaving a 69-bp FRT site and linker sequence precisely in place of the Gli CRM (chr2:113640843-113641280). The Gli binding sites within the CRM were mutated from TAGGTGGTC (chr2:113641085-113641088) to TACCACGTC and from CACCTCCCA (chr2:113641174-113641177) to CACGTGGCA; the mutated CRM was flanked by a 5' *EcoRI* site and a 3' 70-bp linker sequence that included the FRT site. The official name for the *Grem1*<sup>AGRE1</sup> allele is Rr26<tm1Svok> (MGI:5486166). This allele results in the replacement of the 438-bp CRM sequence with an 89-bp sequence containing a single LoxP scar. Further details on the generation of this allele are provided in the supplementary material Fig. S4.

### Embryonic manipulations

When applicable, limbs were cultured for 15 h in 10  $\mu$ M cyclopamine dissolved in absolute ethanol (Toronto Research) or 0.125% absolute ethanol for controls. After incubation, limb buds were separated from adjacent tissues and processed for qRT-PCR,  $\beta$ -galactosidase staining or *in situ* hybridization. All limb buds were assayed for  $\beta$ -galactosidase activity by staining overnight using established methods (Whiting et al., 1991). Skeletal preparations were performed as described previously (Allen et al., 2011).

### Quantitative RT-PCR

RNA was extracted from a single forelimb bud (or paired forelimbs in Fig. 5 and supplementary material Fig. S1) using the RNA-Aqueous 4-PCR Kit (Ambion) and subsequently treated with DNaseI. cDNA was synthesized from 300 ng of total RNA with random hexamers using SuperScript II (Invitrogen). qRT-PCR experiments were performed using Power SYBR Green (Applied Biosystems) on a Viia7 system (Applied Biosystems). Target gene expression was determined by amplifying with the following primer pairs: F-Fmn1: GACGCCGACCAACTTTATG; R-Fmn1: GGCCTCTGACA-GGGGTTTTT; F-Gapdh: GGTGAAGGTCGGTGTGAACG; R-Gapdh: CTCGCTCCTGGAAGATGGTG; F-Gli1: CCCAGCTCGTCCGCAAACA; R-Gli1: CTGCTGCGCATGGCACTCT; F-Grem1: ACTCGTCCACAGCG-AAGAAC; R-Grem1: TCATTGTGCTGAGCCTTGTC; F-Jag1: GTGCTA-CAATCGTGCCAGTG; R-Jag1: GGGGACCACAGACGTTAGAA; F-LacZ: GGGCCGCAAGAAAACATATCC; R-LacZ: TCTGACAATGGCAGATCCCA; F-Shh: TCTCGAGACCAACTCCGAT; R-Shh: GACTTGTCTCCGATCCC-CAC. The *Gapdh* primers are widely used and the *lacZ* primers were previously described (Jeong et al., 2002). Unless specified otherwise (Fig. 5), gene expression levels were normalized to *Gapdh*.

### Statistics

Unless indicated otherwise, statistical significance was measured using Fisher's Exact Test with a two-tailed *P*-value. The compound crosses of mice used to determine genetic interactions were compared with littermate controls.

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### Competing interests

The authors declare no competing financial interests.

### Author contributions

Q.L., J.P.L. and J.L.N. conceived experiments, collected and interpreted data and edited the manuscript. M.B.P. and S.H.C. performed experiments. S.A.V. conceived experiments, interpreted data and wrote the manuscript.

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### Supplementary material

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## Supplemental Table and Figure Legends

**Table S1. *Gli3* and *Gremlin*<sup>ΔGRE1</sup> genetically interact.** Summary of digit phenotypes in E18.5 littermates generated by compound crosses with *Gli3* and *Gremlin*<sup>ΔGRE1</sup>. The presence of nubs was assayed visually upon dissection. All other phenotypes were obtained by inspecting processed skeletal preparations.

**Table S2. Schematic summary of results.** Schematics show representative data for the main experimental results of this study. Arrows indicate polydactylous nubs or digits in skeletal preparations. See Table S1 a complete description of skeletal phenotypes in *Gli3*<sup>+/-</sup>; *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> compound crosses.

**Figure. S1. *Gli1* is significantly reduced in *Gli3*<sup>-/-</sup> forelimbs.** Datapoints indicate relative gene expression assayed by qRT-PCR for pairs of forelimbs from single embryos normalized to a single 32 somite wild-type sample for Wild-type (n=6) and *Gli3*<sup>-/-</sup> littermates (n=5). All embryos were between 32-34 somites. (A) The presence of an asterisk (red) indicates that *Gli1* expression is significantly reduced in *Gli3*<sup>-/-</sup> forelimbs (Mann-Whitney U Test; U=1.000, p = 0.0087). (B) In the same samples, *Shh* levels tend to be reduced although not to statistically significant levels (Mann-Whitney U Test; U=6.000, p = 0.1255). The bars indicate the mean and standard error of mean.

**Figure. S2. *GRE1* enhancer activity in various genetic backgrounds at E11. *GRE1LacZ*<sup>+/-</sup>** forelimbs stained for β-galactosidase activity. Limb buds in A,D,E,F are from embryos that were 40 somites, B from 38 somites, C from 42 somites. ‘a.’, anterior; ‘p.’, posterior.

**Figure. S3. *GRE1* enhancer activity is not negatively regulated by FGF.** E11.5 *GRE1LacZ*<sup>+/-</sup> forelimbs (45-48 somites) were cultured in vehicle-containing control media (0.1% DMSO) (A) while their contralateral forelimbs were cultured in 10μM SU5402 8 hours (B) and stained for β-galactosidase activity. The normalized distance of the β-galactosidase domain from the distal (C) and anterior (D) limb (schematized as red lines in (A)) is not significantly altered in SU5402-treated embryos (Mann Whitney U Test). Horizontal lines indicate the mean and standard error of mean. (E,F) Consistent with previous reports, inhibiting FGF signaling results in an increase in distal anterior *Gremlin* expression (n=2). (G,H) Contralateral hindlimbs from the same embryos shown in panels A and B show a reduction in the FGF target gene *Sprouty4* in SU4202 cultured limb buds (H) compared to contralateral limb buds cultured in control media (G). Images A,B,G,H are from a 48 somite embryo. Images E,F are from a 45 somite embryo.

**Figure S4. Generation of *Gremlin*<sup>ΔGRE1</sup> mice.** (A) A 7.5kb genomic fragment containing the *Gremlin* CRM (chr2:113,637,382-113,644,893) was digested from BAC #RP23-113H17 with *XmaI* and *KpnI* and cloned into a pBluescript upstream of a diphtheria toxin A (DTA) negative selection cassette. A single loxP site and then an FRT-Neo-FRT-LoxP cassette were inserted immediately upstream and downstream, respectively, of the CRM (chr2:113640843-113641280). The targeting vector was linearized with *KpnI* and electroporated into AV3 ES cells (obtained from Dr. Andy McMahon’s laboratory). Approximately 200 colonies were screened by Southern blot. DNA was digested with *Bgl I* and hybridized with a 5’ probe corresponding to chr2:113636128-113636494 and a 3’ probe corresponding mm10l chr2:113,648,199-113,648,558. We identified two correctly targeted colonies by Southern blot, which were used to generate chimeric mice. Germline-transmitting chimeras were crossed with a Cre deleting strain,

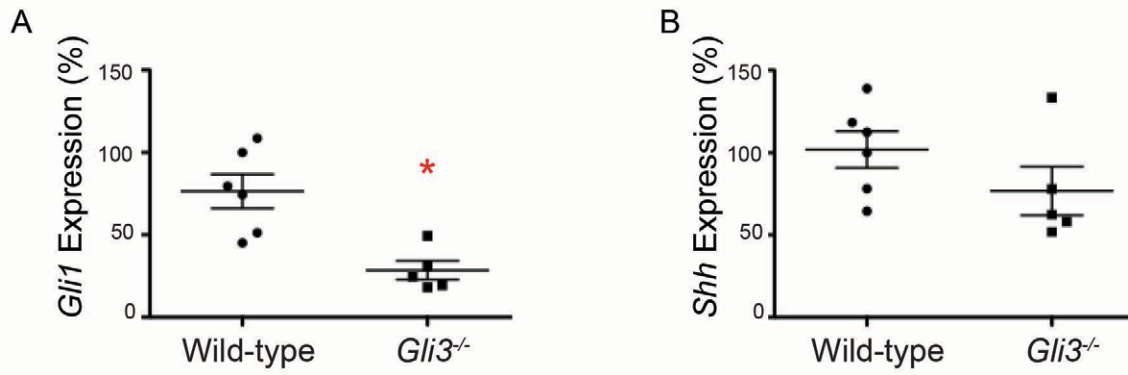
*Sox2Cre*, to generate a deletion allele, *Gremlin*<sup>ΔGRE1</sup> (MGI:5486166). (B) Mice that were wild-type, heterozygous and homozygous null mice were determined by genotyping with primers flanking the CRM (indicated by arrows underneath targeting constructs) that amplify a 582bp fragment in the wild-type allele and a 210bp fragment in the deletion allele. The sequences of the primers are: 5'-GCTAAACACAAAGAAGACTTTTAATGG-3' and 5'-GCAGCAGCAGTATTTTTCAGA-3'.

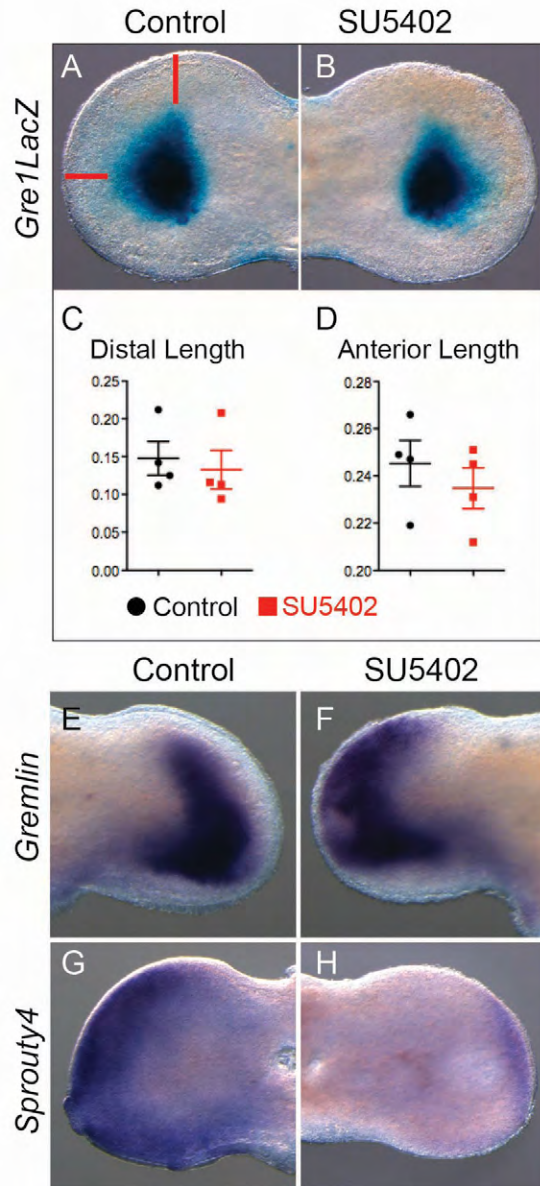
**Figure S5. A single copy of the *Gremlin* with GRE1 is sufficient for limb development.**

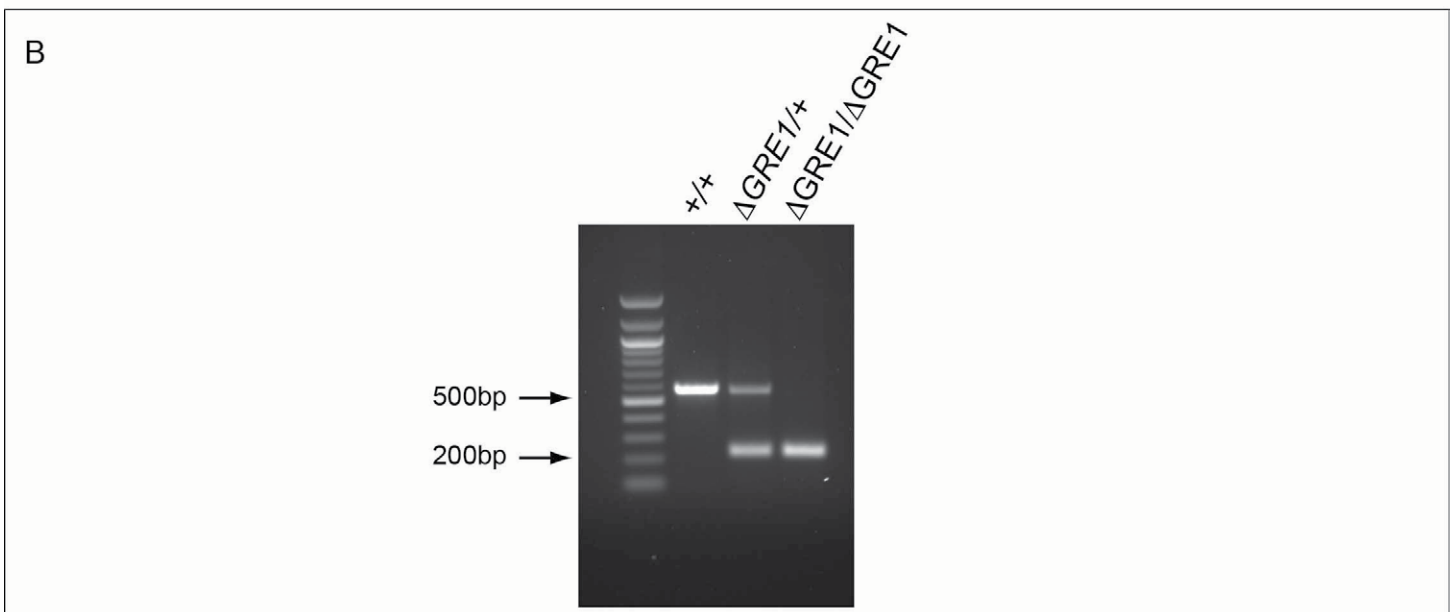
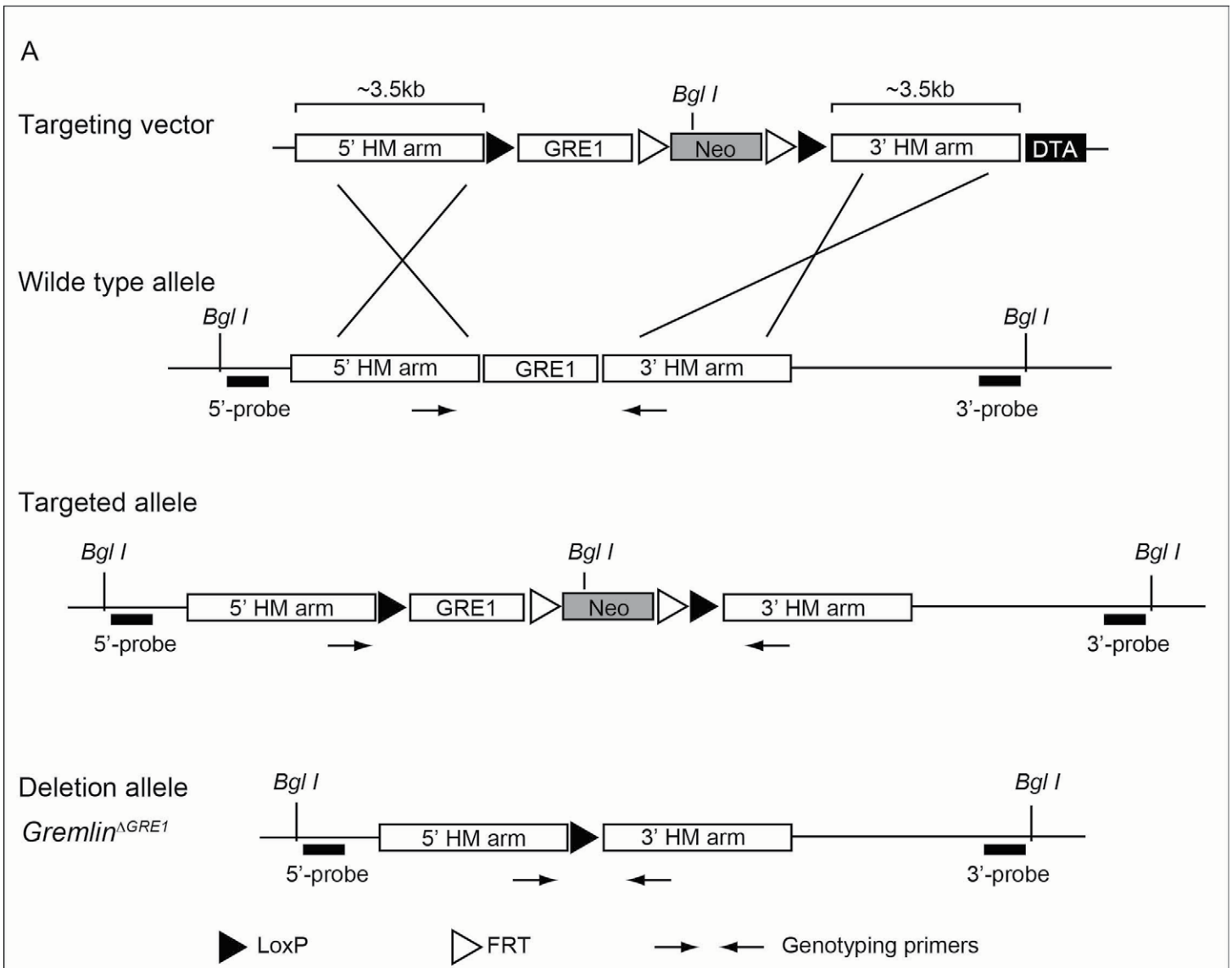
Embryos containing a null allele of *Gremlin* and the other allele with a deletion of the CRM have normal limb skeletal patterning. The *Gremlin* allele is *Greml*<sup>tm1Rmh</sup>. Images depict forelimbs and hindlimbs from the same embryo at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). The numbers of skeletons that were analyzed for each genotype are indicated below.

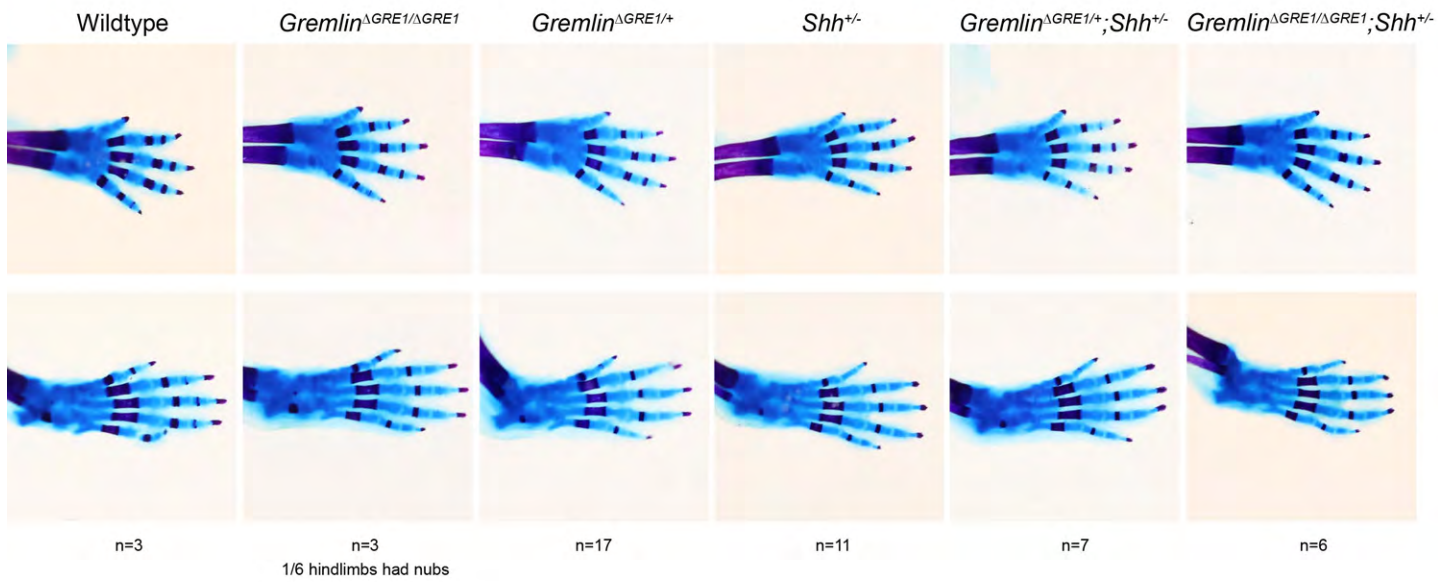
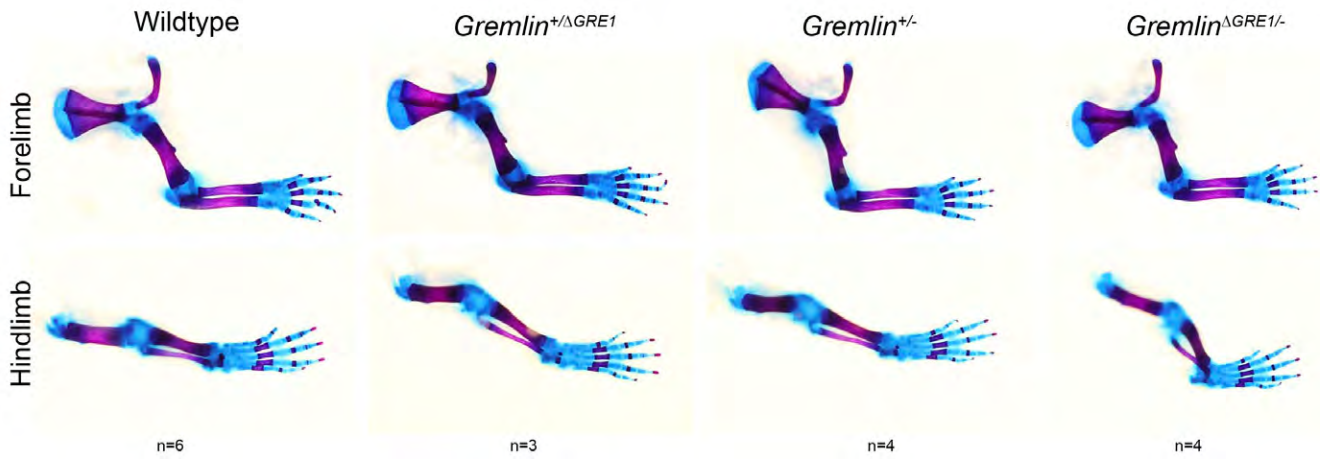
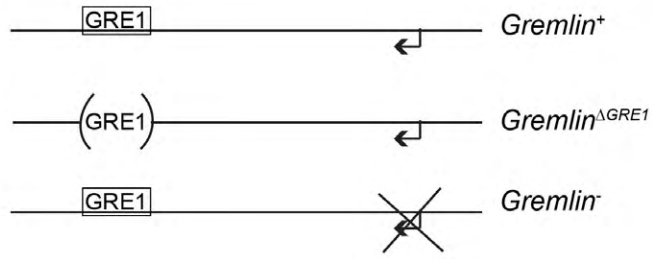
**Figure S6. The *Gremlin*<sup>ΔGRE1</sup> allele does not genetically interact with *Shh*.**

*Gremlin*<sup>ΔCRM/+</sup>; *Shh*<sup>+/-</sup> males were crossed to *Gremlin*<sup>ΔGRE1/+</sup> females and their litters were analyzed by skeletal preparations at E18.5 stained for bone (Alizarin Red) and cartilage (Alcian Blue). Representative hands from forelimbs are shown on the top row. The second row shows representative feet. The numbers of skeletons that were analyzed for each genotype are indicated below. All skeletal preparations were normal with the exception of a small, fleshy nub observed on one hindlimb of a *Gremlin*<sup>ΔGRE1/ΔGRE1</sup> skeleton (something we have never otherwise observed).









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	Wild-Type	<i>Gremlin</i> <sup>Δ<i>GRE1</i>/+</sup>	<i>Gremlin</i> <sup>Δ<i>GRE1</i>/Δ<i>GRE1</i></sup>	<i>Gli3</i> <sup>+/-</sup>	<i>Gremlin</i> <sup>Δ<i>GRE1</i>/+</sup> ; <i>Gli3</i> <sup>+/-</sup>	<i>Gremlin</i> <sup>Δ<i>GRE1</i>/Δ<i>GRE1</i></sup> ; <i>Gli3</i> <sup>+/-</sup>
Forelimbs						
Polysyndactyly	0	0	0	4	23	8
Distal						
Bifurcation	0	0	0	3	3	0
Broad Thumb	0	0	0	3	2	0
Normal	8	38	14	7	0	0
Total	8	38	14	17	28	8
Hindlimbs						
Polydactyly	0	0	0	0	1	3
Nub	0	0	0	18	13	5
Normal	8	38	14	0	0	0
Total	8	38	14	18	14	8



Enhancer Activity					
	Wild-type	<i>Shh</i> <sup>-/-</sup>	<i>RosaSmoM2</i>	<i>Gli3</i> <sup>+/-</sup>	<i>Shh</i> <sup>-/-</sup> ; <i>Gli3</i> <sup>-/-</sup>
<i>GRE1LacZ</i> ( <i>GRE1</i> ::minimal promoter::LacZ)					
E10.5					
E11					
Enhancer activity requires sustained Hh signaling					
BAC Transcriptional Reporter					
BAC with <i>LacZ</i> in <i>Gremlin</i> transcript	Wild-type	<i>GRE1</i> Deletion	<i>GRE1</i> -GliMut		
E11					
GRE1 deletion phenotypes					
	Wild-type	<i>Gremlin</i> <sup>ΔGRE1/ΔGRE1</sup>	<i>Gli3</i> <sup>+/-</sup>	<i>Gremlin</i> <sup>ΔGRE1/ΔGRE1</sup> ; <i>Gli3</i> <sup>+/-</sup>	
In situ hybridization for <i>Gremlin</i>					
E10.5					
Skeletal preparations					
Foot					
Skeletal elements are normal in <i>Gremlin</i> <sup>ΔGRE1/-</sup> and <i>Gremlin</i> <sup>ΔGRE1/ΔGRE1</sup> ; <i>Shh</i> <sup>+/-</sup> embryos					