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Hox5 interacts with *Plzf* to restrict *Shh* expression in the developing forelimb

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To date, only the five most posterior groups of *Hox* genes, *Hox9–Hox13,* have demonstrated loss-of-function roles in limb patterning. Individual paralog groups control proximodistal patterning of the limb skeletal elements. *Hox9* genes also initiate the onset of *Hand2* expression in the posterior forelimb compartment, and collectively, the posterior *HoxA/D* genes maintain posterior *Sonic Hedgehog* (*Shh*) expression. Here we show that an anterior *Hox* paralog group, *Hox5*, is required for forelimb anterior patterning. Deletion of all three *Hox5* genes (*Hoxa5*, *Hoxb5*, and *Hoxc5*) leads to anterior forelimb defects resulting from derepression of *Shh* expression. The phenotype requires the loss of all three *Hox5* genes, demonstrating the high level of redundancy in this *Hox* paralogous group. Further analyses reveal that *Hox5* interacts with *promyelocytic leukemia zinc finger* biochemically and genetically to restrict *Shh* expression. These findings, along with previous reports showing that point mutations in the *Shh* limb enhancer lead to similar anterior limb defects, highlight the importance of *Shh* repression for proper patterning of the vertebrate limb.

limb development | organogenesis | anteroposterior limb patterning | gene interactions | mouse developmental genetics

Limb buds initially emerge as small bulges protruding from the embryonic lateral plate mesenchyme, and development proceeds along three axes: dorsoventral (DV), proximodistal (PD), and anteroposterior (AP) (1). Numerous factors involved in the establishment of these three axes have been defined; for example, DV patterning depends on the antagonism between *Wnt7a* from the dorsal ectoderm and bone morphogenetic protein genes (*BMP*s) and *Engrailed1* (*EN1*) from the ventral ectoderm, growth along the PD axis is regulated mainly by fibroblast growth factor genes (*Fgf*s) secreted from the apical ectodermal ridge (AER), and establishment of the AP axis requires signaling from a region of the posterior limb bud termed the zone of polarizing activity (ZPA). *Sonic Hedgehog* (*Shh*) is the morphogen secreted from this region (2), and loss of *Shh* function results in the absence of posterior limb elements (3, 4). Previous research has identified a limb-specific enhancer located in the fifth intron of *limb region 1 protein homolog* gene appeoximately 1 Mb from the *Shh* coding sequence, designated the ZPA regulatory sequence (ZRS) (5). Deletion of this enhancer leads to defects similar to *Shh* loss-of-function mutants (6–8).

Hox genes also have been shown to play pivotal roles in limb PD patterning of the limb skeletal elements. The *HoxA* and *HoxD* genes from groups 9–13 impact forelimb development along the PD axis (9–14). *Hoxa9/d9* and *Hox10* paralogs specify stylopod patterning (humerus and femur) (10, 14, 15). Loss of function of *Hoxa11* and *Hoxd11* results in dramatic mispatterning of the zeugopod (radius/ulna and tibia/fibula) (9, 14). Loss of autopod elements (i.e., handplate and footplate) in *Hoxa13/d13* mutants reveals important roles for this group in autopod patterning (11).

In addition, the *HoxA/D9–13* paralogous group genes are collectively required for the activation and maintenance of *Shh* expression in limb AP patterning (12, 13). Although misexpression of more anterior *Hox* genes in mice reportedly affects limb patterning (16), no loss-of-function mutants of anterior, non–*abdominal B* (*AbdB*)-related genes have demonstrated defects in the patterning of limb skeletal elements. Moreover, no *HoxB* or *HoxC* group genes had been shown to play a role in forelimb development until a report by our group demonstrated that all four *Hox9* paralogous genes (*Hoxa9*, *Hoxb9*, *Hoxc9*, and *Hoxd9*) are required in the early lateral plate mesoderm to define the posterior forelimb field by regulating the onset of *Hand2* expression (15).

Numerous human syndromes and mouse mutants that affect AP limb patterning have been identified. Disruption of *Shh* expression accounts for some of these phenotypes. Some mutations in the *Shh* limb enhancer ZRS lead to loss of posterior digits reminiscent of loss of *Shh* function (3, 4, 8, 17). In addition, many point mutations in the ZRS identified in spontaneous mouse mutants (*Hx* and *M100081*) (18), human patients (PPD2, Cuban mutation, Werner mesomelic syndrome, and others) (5–7, 18–27), chickens (17), and cats (28) that lead to anteriorized and/or ectopic expression of *Shh,* indicating that the ZRS enhancer not only directs activation of *Shh* in the ZPA, but also is responsible for repression of *Shh* in the anterior limb.

Significance

Mammalian *Hox* genes are important for limb development. Posterior *abdominal B* (*AbdB*) *Hox* groups (*Hox9–Hox13*) are required for establishment of the limb proximodistal axis. In addition, *Hox9* genes control the onset of *Hand2* expression in the posterior forelimb, and *HoxA/D AbdB* genes are responsible for the initiation and maintenances of *Sonic Hedgehog* (*Shh*). In this study, we generated *Hox5* triple mutants, resulting in embryos with severe forelimb anterior patterning defects. We found that Hox5 proteins interact with *promyelocytic leukemia zinc finger* to restrict *Shh* expression in the forelimb bud. The hindlimb in *Hox5* mutants develops normally, revealing distinct differences in anteroposterior field establishment in the forelimb and hindlimb and unanticipated roles for non-*AbdB Hox* genes, including *HoxB* and *HoxC* group genes, in limb development.

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Mutations in factors that have not been associated with *Shh* signaling also can lead to anterior limb defects. For example, patients with Holt–Oram syndrome (HOS) and Okihiro syndrome (OS), which are caused by mutations of *TBX5* and the *Spalt* family zinc finger transcription factor *SALL4*, respectively, show anterior forelimb defects, including loss of a thumb or a triphalangeal digit and/or hypoplasia of the radius (29–36). Loss of function of promyelocytic leukemia zinc finger gene (*Plzf*) function in both human patients and mouse mutants also results in similar limb AP patterning defects (37–39). Interactions among the myriad of required factors in limb AP patterning and their relationships to *Shh* signaling and other signaling pathways remain incompletely understood.

In this study, we demonstrate that *Hox5* genes perform a novel function in limb AP patterning. Loss of function of *Hox5* paralogous genes (*Hoxa5*, *Hoxb5*, and *Hoxc5*), an anterior set of *Hox* genes not belonging to the *AbdB*-related *Hox* group, results in defects in anterior forelimb patterning that closely resemble some point mutations in the ZRS in both mice and humans. Early pattering of the anterior and posterior limb compartments is not disrupted in these mutants; however, the limb defects in *Hox5* mutants are associated with ectopic *Shh* expression in the anterior forelimb buds, and we provide molecular and genetic evidence indicating that *Hox5* interacts with *Plzf* to restrict *Shh* expression and pattern the anterior forelimb.

Results

Inactivation of *Hox5* Paralogous Group Genes Results in Anterior Forelimb Defects. Single mutants for *Hoxa5*, *Hoxb5*, and *Hoxc5* (the three mammalian *Hox5* paralogous group genes) have been generated previously (40–42). Although loss of *Hoxa5* function results in a smaller scapula (43), no limb patterning abnormalities have been reported for any of the three *Hox5* single mutants despite the expression of these genes in the developing forelimb and hindlimb (42, 43) (Fig. S1). Compound mutants deficient for any combination of as many as five of the six *Hox5* alleles did not exhibit limb defects (Fig. 1 *A* and *I*). Only when all six *Hox5* alleles were mutated were defects in the anterior forelimb skeletal elements observed (Fig. 1 *A*–*H*). The humerus of *Hox5* triple mutants was variably affected (Fig. 1 *B* and *C*), the radius was truncated or lost (Fig. 1 *B* and *C*), and digit 1 was often missing or transformed into a triphalangeal digit, with the distal portion of digit 2 occasionally bifurcated (Fig. 1 *E*–*G*). Hindlimb development was not affected in *Hox5* mutant mice, even though *Hox5* was expressed at early hindlimb bud stages (Fig. 1 *J* and *K*).

Shh Is Ectopically Activated in *Hox5* Mutant Forelimb Buds. Given the clear disruption of AP limb patterning and the importance of *Shh* in this process, we examined *Shh* expression in *Hox5* mutants. *Shh* expression expanded anteriorly in early forelimb buds of *Hox5* mutants, and ectopic *Shh* expression was observed in anterior domains in some instances (Fig. 2 *A*–*E*). The expression of downstream factors *Ptch1* and *Gli1* was consistently anteriorized in *Hox5* triple-mutant embryos (Fig. 2 *F*, *G*, *J*, and *K*). *Fgf4* expression in the AER extended anteriorly compared with controls (Fig. 2 *H* and *L*), consistent with anteriorized *Shh* expression, whereas *Fgf8* was expressed normally in the AER (Fig. 2 *I* and *M*).

Because *Shh* expression is disrupted at early stages, we examined AP patterning regulators upstream of *Shh* signaling. In somite-matched *Hox5* mutants and controls, there were no observable differences in the expression of *Gli3* (Fig. 3 *A*, *C*, *F*, and *H*) or *Hand2* (Fig. 3 *B*, *D*, *G*, and *I*) at any stage examined. *Alx4*, another early regulator of anterior limb patterning, was expressed normally in *Hox5* mutants (Fig. 3 *E* and *J*).

Misexpression of *HoxD* genes results in preaxial polydactyly phenotypes bearing some resemblance to *Hox5* mutants (12, 13, 16, 44), and ectopic or anteriorized *Shh* expression also leads to coincident anteriorization of *Hoxd10-13* (45). Expression of

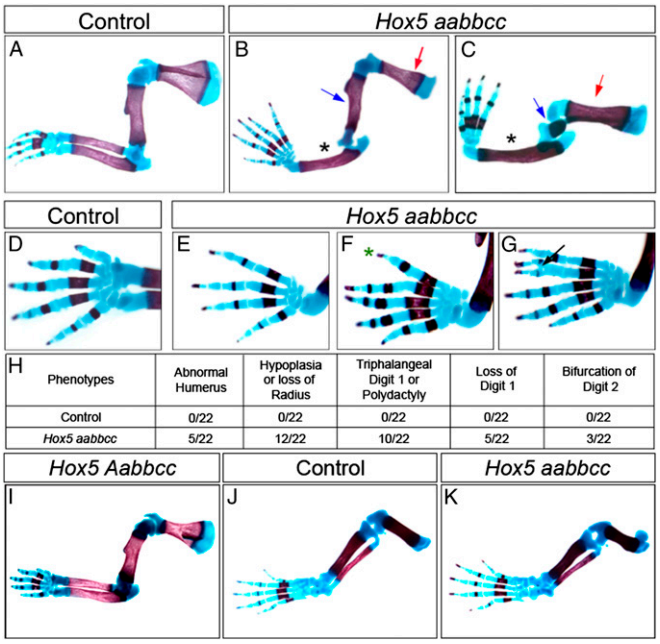

Fig. 1. Loss of function of *Hox5* paralogous genes results in anterior forelimb defects. (*A*–*G*) Skeletal analysis of control and *Hox5* triple-mutant forelimbs at E18.5. The scapula is reduced in *Hox5* triple mutants compared with controls, as observed for *Hoxa5* single mutants (*A*–*C*, red arrows). The stylopod is reduced or truncated only in embryos with a phenotype in the radius (*B* and *C*, blue arrow). The radius of mutant forelimbs is missing or severely truncated (*B* and *C*, black asterisk). (*D*–*G*) The most anterior digit develops abnormally in *Hox5* mutants compared with controls. Digit 1 is often missing (*E*) or triphalangeal (*F* and *G*, green asterisk). Less frequently, *Hox5* mutants also have a bifurcated digit 2 (*G*, black arrow). Digit phenotypes do not correlate with the severity of stylopod/zeugopod defects. (*H*) Table summarizing forelimb phenotypes of *Hox5* mutant forelimbs. (*I*) Compound mutants deficient for as many as five of the six *Hox5* alleles do not exhibit limb defects. (*J* and *K*) Hindlimb development is not affected in *Hox5* mutants.

Hoxd10-13 genes in *Hox5* mutants was shifted anteriorly in *Hox5* mutant forelimbs at embryonic day (E) 10.5 (Fig. 3 *K*–*R*), consistent with misregulation of *Shh.* It is important to note that *HoxD* genes are not linked to the *HoxA*, *HoxB*, or *HoxC* clusters, and thus these effects cannot be due to *cis* effects from the targeted mutations introduced into the *Hox5* alleles.

***Plzf* Is a Potential Coregulator of *Shh* Repression.** Several additional regulators of anterior limb patterning have been identified in human disease syndromes as well as in mouse mutants. Forelimb defects similar to those seen in *Hox5* mutants have been identified in patients with HOS caused by *Tbx5* mutations (32–36), and *Hox* genes have been reported to be capable of driving *Tbx5* expression (46), OS caused by *Sall4* mutations (29, 30), Townes–Brocks syndrome caused by *Sall1* mutations (31, 47) and Saethre–Chotzen syndrome caused by *Twist1* mutations, which also show limb phenotypes in mutant mice (48–50). Mutations of both the human and mouse limb enhancer of *Shh*, ZRS (7, 18, 19, 24), and human multiple congenital anomaly/mental retardation syndromes caused by mutations of *Plzf*, as well as loss-of-function mutations in *Plzf* in mice (37, 39), lead to similar phenotypes. Based on these similarities, we investigated the expression of *Tbx5, Sall1*, *Sall4, Twist1*, and *Plzf* in our *Hox5* mutants. We found no change in the expression of any of these genes in *Hox5* mutant forelimbs (Figs. S2 *A*–*L* and S3 *A*–*D*).

To test whether these factors might act in parallel in the same pathway as *Hox5* and affect *Shh* expression, we examined *Shh*

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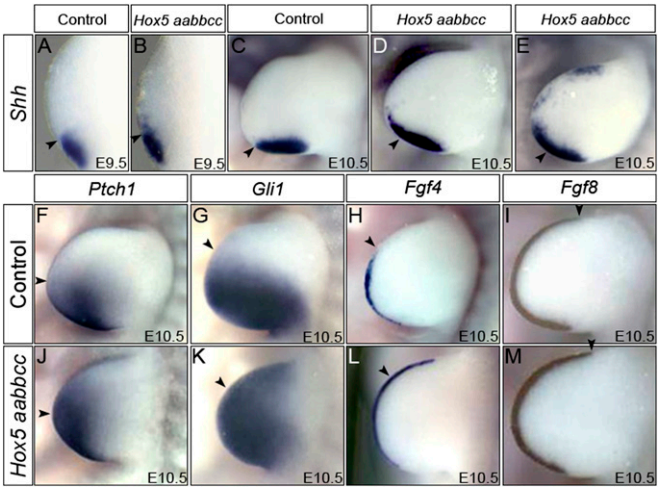


Fig. 2. *Shh* signaling is disrupted in *Hox5* mutant forelimbs. (*A–E*) *Shh* expression is anteriorized in *Hox5* mutant forelimbs. At E9.5, *Hox5* mutant forelimbs display slightly anteriorized *Shh* expression compared with controls (*A* and *B*). Anteriorization of *Shh* expression is observed by E10.5 in *Hox5* mutant forelimbs compared with controls (*C–E*), and ectopic *Shh* expression appears in anterior regions of some mutant forelimbs (*E*). (*F–M*) Expression of *Ptch1* and *Gli1* is consistently shifted anteriorly in *Hox5* mutants at E10.5 compared with controls (*F, J, G,* and *K*). Fgf4 expression is also anteriorized in *Hox5* mutant forelimbs compared with controls (*H* and *L*), whereas *Fgf8* expression is unchanged (*I* and *M*). Black arrowheads in each panel mark the WT anterior boundary of expression for each probe.

expression in *Tbx5* heterozygotes with or without loss of *Sall4* function, as well as in *Plzf* mutant embryos. Consistent with previous reports that *Shh* expression is not altered with loss of *Tbx5* function (51), we found that *Shh* was not altered in *Tbx5* or *Sall4* heterozygous mutants or compound *Tbx5*/*Sall4* heterozygous mutants (Fig. S3 *I–L*); however, *Plzf* mutants showed a small but reproducible anterior shift in *Shh* expression (Fig. 4 *A* and *B*). In addition, *Shh* transcripts were increased by ∼50% in *Plzf* mutant limbs (Fig. 4*C*). To confirm changes in *Shh* expression, we examined the expression of *Ptch1* and *Gli1,* factors immediately downstream of *Shh.* In *Plzf* mutants, *Ptch1* and *Gli1* were consistently anteriorized and ectopically expressed (Fig. 4 *D–G*), demonstrating that *Shh* expression is affected downstream of *Plzf* in the developing limb.

Hox5 Proteins Interact with *Plzf* and Can Regulate *Shh* Expression Through the ZRS in Vitro. *Plzf* mouse mutants have been found to have similar forelimb phenotypes as *Hox5* mutant mice, although with low penetrance (37). The mice used in the present study demonstrated a similar phenotype (Fig. 5 *A* and *B*), but with 100% penetrance in the forelimb (52). Heterozygous embryos had no limb phenotype (Fig. 5*C*). Having already demonstrated no changes in *Plzf* expression in our *Hox5* mutants, we investigated the possibility that *Hox5* acts downstream of *Plzf* in forelimb AP patterning, but found normal *Hox5* expression levels in *Plzf* mutant forelimbs (Fig. S3 *E–G*).

To test whether Hox5 and Plzf proteins are capable of interacting to regulate downstream limb target genes, we examined potential physical interactions between these proteins in vitro. In cells cotransfected with epitope-tagged Hoxa5, Hoxb5, or Hoxc5 protein in combination with tagged Plzf, we found coprecipitation of all three Hox5 proteins with Plzf protein (Fig. 4*H* and Fig. S3*H*), consistent with the possibility that these proteins interact to regulate downstream targets.

If *Hox5* and *Plzf* function together to repress *Shh* expression anteriorly and affect AP patterning of the forelimb, then we would expect these genes to interact in vivo. *Hox5* mutants demonstrate no forelimb defects unless all six alleles of the three *Hox5* genes are mutated (compare Fig. 1 *A–G* and Fig. 5*D*). *Plzf* mutants exhibited forelimb phenotypes only with loss of both alleles, whereas heterozygous animals had no phenotype (Fig. 5 *A–C*). Compound mutants heterozygous for *Plzf* combined with either one or two mutant *Hox5* alleles did not exhibit any forelimb defects. Embryos heterozygous for *Plzf* plus three *Hox5* mutant alleles resulted in preaxial limb skeletal defects in only 1 of 18 forelimbs (Fig. 5*G*). Compound mutants heterozygous for *Plzf* plus four *Hox5* mutant alleles showed more severe forelimb defects with higher penetrance (Fig. 5 *E* and *G*). Forelimb defects were further exacerbated in compound mutants heterozygous for *Plzf* plus five *Hox5* mutant alleles, with 12 of 14 forelimbs demonstrating anterior forelimb defects (Fig. 5 *F* and *G*). These findings indicate that strong genetic interactions occur between *Hox5* and *Plzf* in vivo, supporting the hypothesis that these proteins cooperatively regulate forelimb AP patterning events.

If *Hox5* and *Plzf* coordinately regulate *Shh* expression, then the phenotypes observed in the compound mutants should result in changes in *Shh* pathway expression. Obvious anteriorization of *Shh* expression was observed in *Plzf*/*Hox5* compound mutants (Fig. 5 *H* and *I*), similar to that seen in both *Hox5* triple mutants and *Plzf* mutants, but not in *Plzf* heterozygotes or in compound *Hox5* mutants carrying up to five mutant alleles. *Gli1* and *Ptch1* expression also was anteriorized in the forelimb of the compound *Hox5*/*Plzf* mutants (Fig. 5 *J–M*), but not in control WT embryos, *Plzf* heterozygotes, or compound *Hox5* mutants harboring up to five mutant alleles. These genetic data further support the assertion that *Hox5* and *Plzf* cooperatively repress

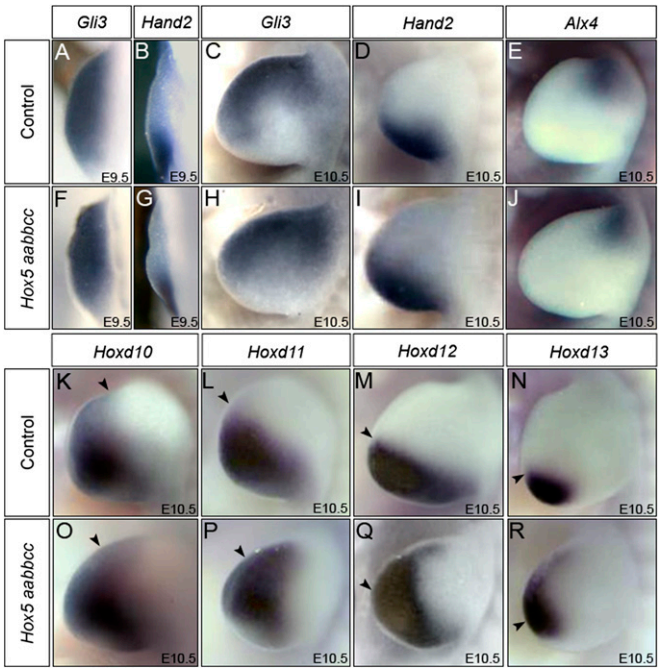


Fig. 3. Early limb patterning pathways are not disrupted, but posterior *HoxD* gene expression is anteriorized in *Hox5* mutants. (*A–J*) Early AP patterning factors are not disrupted in *Hox5* mutants. The expression of *Gli3* is not altered at E9.5 (*A* and *F*) or E10.5 (*C* and *H*). *Hand2* is expressed normally in *Hox5* mutant forelimbs at E9.5 (*B* and *G*) and E10.5 (*D* and *I*). *Alx4* expression in *Hox5* mutants is also comparable to that in controls at E10.5 (*E* and *J*). (*K–R*) The expression limits of posterior *HoxD* genes are anteriorized in E10.5 *Hox5* mutant forelimbs. *Hoxd10* (*K* and *O*), *Hoxd11* (*L* and *P*), *Hoxd12* (*M* and *Q*), and *Hoxd13* (*N* and *R*) are all expressed more anteriorly in *Hox5* mutant forelimb buds (*O–R*) compared with controls (*K–N*). Black arrowheads mark the WT anterior boundary of expression for each probe.

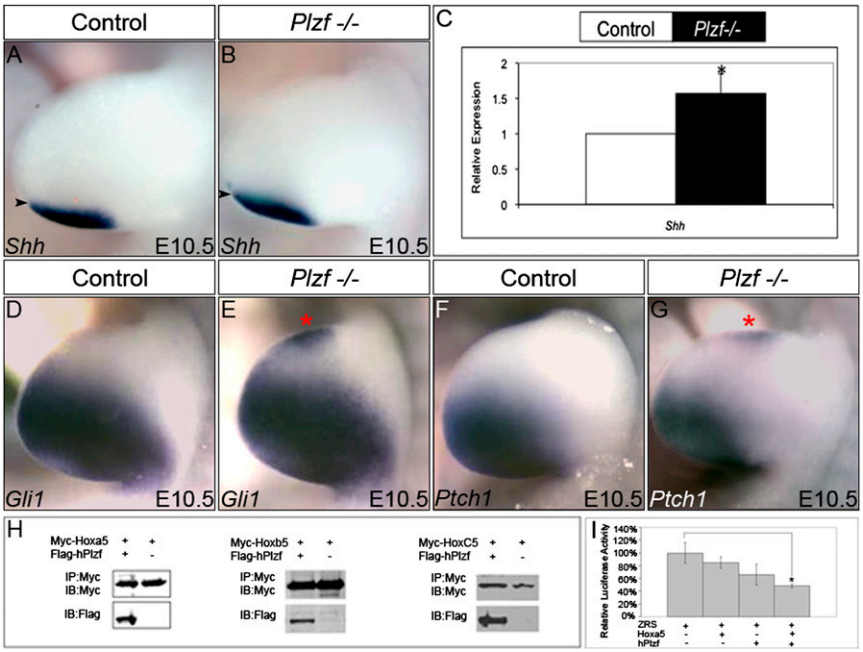
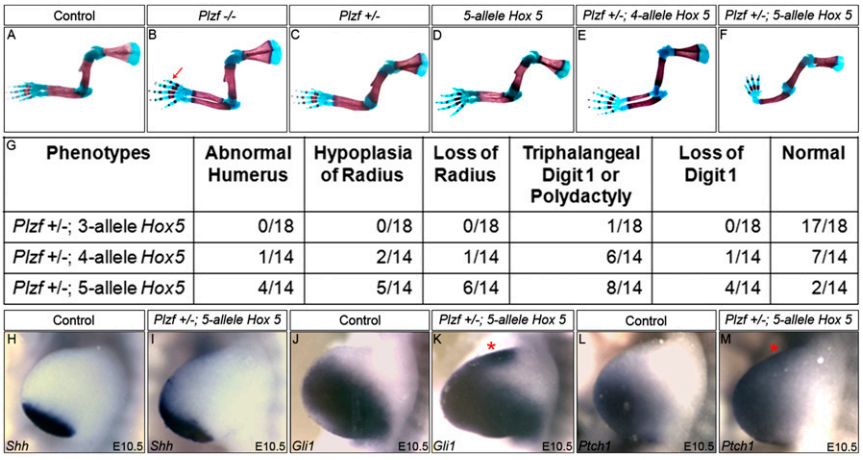


Fig. 4. Hox5 and Plzf cooperatively mediate repression of *Shh* expression via ZRS. (*A*–*G*) The *Shh* signaling pathway is disrupted in *Plzf* mutant forelimbs similar to that in *Hox5* mutant forelimbs. The expression of *Shh* is anteriorized in *Plzf* mutant forelimb buds compared with controls (*A* and *B*; black arrowheads mark the WT anterior boundary), and qRT-PCR analysis demonstrates an increase in *Shh* levels in *Plzf* mutants (*C*; asterisk indicates significant differences from controls; $P < 0.05$). Ectopic, anteriorized *Gli1* and *Ptch1* expression is observed in all *Plzf* mutant forelimbs at E10.5, confirming anteriorized *Shh* activity (*D*–*G*; red asterisks mark ectopic anterior expression). (*H*) Coimmunoprecipitation assays from cell lysates cotransfected with Myc-tagged Hox5 proteins and Flag-tagged Plzf protein. Immunoprecipitation with anti-Myc (Hox) antibodies and immunoblotting (IB) for anti-Flag (Plzf) results in coimmunoprecipitation (IP) of Myc-tagged Hoxa5, Hoxb5, and Hoxc5 with Plzf. (*I*) ZRS-driven luciferase reporter activity trends downward when cotransfected with Hox5 protein expression constructs or with the Plzf protein expression construct; however, cotransfections of the ZRS reporter with both Hox5 and Plzf proteins result in statistically significant down-regulation of expression from this reporter.

anterior *Shh* expression during forelimb development to influence forelimb AP patterning.

To provide evidence supporting possible direct regulation at the ZRS, we examined the ability of transfected Hox5 proteins and Plzf to regulate reporter expression through the ZRS enhancer. Transfection of any of the three Hox5 proteins or Plzf alone with the ZRS reporter did not result in any statistically significant change in expression, although the levels trended downward; however, transfection of any of the Hox5 proteins and Plzf together resulted in a statistically significant down-regulation of baseline expression, consistent with direct regulation of *Shh* expression through the ZRS enhancer (Fig. 4*I*). We made multiple



G Phenotypes	Abnormal Humerus	Hypoplasia of Radius	Loss of Radius	Triphalangeal Digit 1 or Polydactyly	Loss of Digit 1	Normal
Plzf +/-; 3-allele *Hox5*	0/18	0/18	0/18	1/18	0/18	17/18
Plzf +/-; 4-allele *Hox5*	1/14	2/14	1/14	6/14	1/14	7/14
Plzf +/-; 5-allele *Hox5*	4/14	5/14	6/14	8/14	4/14	2/14

Fig. 5. *Hox5* and *Plzf* genetically interact to pattern the anterior limb and *Shh* expression in vivo. (*A*–*G*) Skeletal preparations from E18.5 *Plzf*, 5-allele *Hox5*, and compound *Hox5;Plzf* mutants. *Plzf* heterozygotes and embryos with up to five mutant *Hox5* alleles (*Hox5Aabbcc*) are indistinguishable from controls (*C* and *D*), whereas *Plzf* mutants exhibit preaxial defects with 100% penetrance in our background (*B*; arrow denotes triphalangeal digit 1). Compound mutants heterozygous for *Plzf* plus three mutant *Hox5* alleles rarely display a phenotype (*G*), but *Plzf* heterozygotes with four or five mutant *Hox5* alleles display increases in the penetrance of anterior limb defects (*E*–*G*), demonstrating strong genetic interaction between *Hox5* and *Plzf*. (*H*–*M*) The *Shh* pathway is derepressed in *Hox5;Plzf* compound mutants. *Shh* (*H* and *I*), *Gli1* (*J* and *K*), and *Ptch1* (*L* and *M*) are ectopically expressed in *Plzf* $^{+/-}$*;Hox5* 5-allele compound mutants compared with controls. Controls include WT, Plzf heterozygous, and compound Hox5 mutants with five or fewer mutant alleles; red asterisks denote anteriorized expression.

attempts to perform ChIP of both Plzf and Hox5 at the ZRS promoter in vivo. Despite the successful use of the antibodies at other enhancers, the very high level of background (control antibody) binding at the ZRS and the potentially small numbers of responding cells in the limb bud precluded conclusive evidence of binding of these proteins in vivo (Fig. S4 and *SI Materials and Methods*).

Discussion

In this study, we show a unique and unexpected role for *Hox5* genes in limb AP patterning. A myriad of genetic studies have defined important roles for *HoxA* and *HoxD* group 9–13 genes in forelimb development (9–14). Recently, we reported that *Hox9* genes from *HoxB* and *HoxC* complex, along with *Hoxa9* and *Hoxd9*, are also required to define the posterior forelimb compartment (15). However, previous loss-of-function studies have provided no evidence that anterior, non-*AbdB Hox* genes participate in patterning limb skeletal elements. Here we report limb phenotypes resulting from loss of *Hox5* paralogous gene function, further demonstrating pivotal roles for *HoxB* and *HoxC* complex genes in forelimb AP patterning, with *Hoxa5*, *Hoxb5*, and *Hoxc5* controlling anterior forelimb patterning.

The limb defects in our *Hox5* mutants are restricted to forelimbs, with no defects in hindlimb development observed. The limb defects in quadruple *Hox9* mutants are also restricted to the forelimbs (15). Taken together, our findings highlight significant differences in how anterior and posterior limb compartments are established in forelimbs and hindlimbs. This is surprising, considering the downstream factors currently known to play critical roles in AP patterning (i.e., *Hand2*, *Gli3*, and *Shh*) function similarly in both forelimbs and hindlimbs. Our findings from both loss of *Hox9* paralog function (15) and the present study suggest that early axial *Hox* expression in the lateral plate mesoderm controls the establishment of the anterior (*Hox5*) and posterior (*Hox9*) compartments of the forelimb. None of the numerous combinations of posterior *Hox* loss-of-function mutants reported to date (9–14) are known to lead to hindlimb AP patterning defects analogous to those reported for loss of *Hox9* or *Hox5* paralogs. Itou et al. (53) recently demonstrated that LIM-homeodomain factor *Islet1* is a critical regulator of *Hand2* expression and the posterior compartment in hindlimbs. How the hindlimb anterior compartment is established remains to be discovered.

It is also interesting to note that although *Hox5* paralogs and *Hox9* paralogs control anterior and posterior patterning, respectively, *Hox9* paralogs are responsible for initiation of *Hand2*, whereas no disruption of early anterior/posterior compartment formation is observed in *Hox5* mutants (ref. 15 and this paper). In *Hox5* mutant limbs, the initial *Hand2*/*Gli3* pattern is normal, but downstream expression of *Shh* is affected, consistent with *Hox5* regulating *Shh* expression more directly. This finding is also consistent with previous reports demonstrating that numerous point mutations in the ZRS of mouse, humans, chickens, and cats result in ectopic *Shh* activity in anterior domains of the limb bud and result in phenotypes similar to those that we detected in *Hox5* mutant mice, including defects in the stylopod, anterior zeugopod, and digits (5–7, 17–28).

The findings reported here also reveal a role for *Plzf* in regulating *Shh* expression in limb AP patterning. We detected anteriorized *Shh* expression in *Plzf* mutants, in contrast to a previous report (37). The discrepancy may be related to the use of different *Plzf* mutant alleles; we observed 100% penetrance of forelimb defects in the mutants used in the present study, significantly higher than in the previously reported mutant allele (37). Our finding that *Shh* is regulated downstream of *Plzf* in limb AP patterning is further supported by changes in *Ptch1* and *Gli1* expression in addition to *Shh* expression.

Our genetic and molecular analyses of *Hox5* function in forelimb development support a model in which Hox5 proteins, interacting with Plzf, act as repressors of *Shh* expression. Among the many potential binding sites in the ZRS are several putative Hox- binding sites and at least one putative Plzf-binding site. The putative Plzf site is mutated in the "Cuban mutation," one of the human mutations with limb phenotypes similar to those in the mice reported here, including radial aplasia (7). There is also a report of three independent probands with anterior forelimb phenotypes (mostly triphalangeal thumbs) that harbor a mutation in one of the three putative Hox-binding sites (19).

The activity of Hox5 and Plzf likely combine with numerous other factors that bind to this enhancer to both activate and repress *Shh* expression. Several factors, including *Hand2* and *HoxD*, have been shown to activate *Shh* expression via the ZRS limb enhancer element (54, 55). *Etv4*/Etv5 and *Twist 1* have been shown to cooperate to restrict *Shh* expression to the posterior limb bud (50, 56). The ZRS is more than 700 bp long, and thus it is likely that a myriad of factors converge at this critical regulatory hub to direct proper expression of *Shh* in the developing vertebrate limb. A complete understanding of the factors that bind to these sites and how they interact remains to be delineated in future studies.

Materials and Methods

Mice and Whole-Mount in Situ Hybridization. All mouse mutant strains used in this study have been reported previously (41, 52). Control mice included both WT embryos and low-allele littermates from *Hox* and *Hox*/*Plzf* crosses. The results were identical, and thus we use the term "control" throughout for clarity. Mutant mouse strains, early skeletal preparations, and standard whole-mount in situ hybridization were as described previously (14, 41, 52). All in situ probes were prepared as described previously (15, 57, 58). All experiments were performed following protocols approved by the University of Michigan's Institutional Committee on the Use and Care of Animals.

Cell Culture, Transfections, Luciferase Assays, and Coimmunoprecipitation Assays. HEK293 or HEK293T cells were used and plated as described previously (59). Cell transfections were performed by $CaPO_4$ precipitation. Coimmunoprecipitation assays were performed as described previously (59). Hox5 and Plzf protein-coding sequences were amplified from their cDNAs using the primers listed in *SI Materials and Methods*, then subcloned into pCS2+MT or p3XFlag-CMV vectors (Sigma-Aldrich). Details of plasmid generation and reporter assays have been reported previously (59). The highly conserved ZRS core region was amplified using the primers listed in *SI Materials and Methods* and then subcloned into a pGL3 promoter vector (Promega). The Student *t* test was used to determine statistical significance. All experiments were repeated at least three times in independent experiments.

RNA Isolation and Quantitative RT-PCR. RNA was isolated from mouse limbs with the Qiagen RNeasy Micro Kit. Quantitative RT-PCR (qRT-PCR) was carried out using Roche FastStart SYBR Green Master Mix. Primer sequences have been described previously (51). Relative expression values were calculated as $2^{-\Delta\Delta Ct}$, and values of controls were normalized to 1. GAPDH served as an internal control for normalization in all qRT-PCR experiments, and the Student *t* test was used to determine statistical significance ($P < 0.05$). All experiments were repeated at least three times.

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1. Towers M, Tickle C (2009) Generation of pattern and form in the developing limb. *Int J Dev Biol* 53(5-6):805–812.
2. Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75(7):1401–1416.
3. Chiang C, et al. (2001) Manifestation of the limb prepattern: Limb development in the absence of sonic hedgehog function. *Dev Biol* 236(2):421–435.
4. Kraus P, Fraidenraich D, Loomis CA (2001) Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech Dev* 100(1):45–58.

5. Lettice LA, et al. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* 12(14):1725–1735.
6. Wieczorek D, Köster B, Gillessen-Kaesbach G (2002) Absence of thumbs, A/hypoplasia of radius, hypoplasia of ulnae, retarded bone age, short stature, microcephaly, hypoplastic genitalia, and mental retardation. *Am J Med Genet* 108(3):209–213.
7. Zguricas J, et al. (1999) Clinical and genetic studies on 12 preaxial polydactyly families and refinement of the localisation of the gene responsible to a 1.9 cM region on chromosome 7q36. *J Med Genet* 36(1):32–40.
8. Sagai T, Hosoya M, Mizushina Y, Tamura M, Shiroishi T (2005) Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb. *Development* 132(4):797–803.
9. Davis AP, Witte DP, Hsieh-Li HM, Potter SS, Capecchi MR (1995) Absence of radius and ulna in mice lacking hoxa-11 and hoxd-11. *Nature* 375(6534):791–795.
10. Fromental-Ramain C, et al. (1996) Specific and redundant functions of the paralogous Hoxa-9 and Hoxd-9 genes in forelimb and axial skeleton patterning. *Development* 122(2):461–472.
11. Fromental-Ramain C, et al. (1996) Hoxa-13 and Hoxd-13 play a crucial role in the patterning of the limb autopod. *Development* 122(10):2997–3011.
12. Kmita M, et al. (2005) Early developmental arrest of mammalian limbs lacking HoxA/HoxD gene function. *Nature* 435(7045):1113–1116.
13. Tarchini B, Duboule D, Kmita M (2006) Regulatory constraints in the evolution of the tetrapod limb anterior-posterior polarity. *Nature* 443(7114):985–988.
14. Wellik DM, Capecchi MR (2003) Hox10 and Hox11 genes are required to globally pattern the mammalian skeleton. *Science* 301(5631):363–367.
15. Xu B, Wellik DM (2011) Axial Hox9 activity establishes the posterior field in the developing forelimb. *Proc Natl Acad Sci USA* 108(12):4888–4891.
16. Knezevic V, et al. (1997) Hoxd-12 differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* 124(22):4523–4536.
17. Maas SA, Suzuki T, Fallon JF (2011) Identification of spontaneous mutations within the long-range limb-specific Sonic hedgehog enhancer (ZRS) that alter Sonic hedgehog expression in the chicken limb mutants oligozeugodactyly and silkie breed. *Dev Dyn* 240(5):1212–1222.
18. Sagai T, et al. (2004) Phylogenetic conservation of a limb-specific, cis-acting regulator of Sonic hedgehog (Shh). *Mamm Genome* 15(1):23–34.
19. Furniss D, et al. (2008) A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb. *Hum Mol Genet* 17(16):2417–2423.
20. Albuisson J, et al. (2011) Identification of two novel mutations in Shh long-range regulator associated with familial pre-axial polydactyly. *Clin Genet* 79(4):371–377.
21. Farooq M, et al. (2010) Preaxial polydactyly/triphalangeal thumb is associated with changed transcription factor-binding affinity in a family with a novel point mutation in the long-range cis-regulatory element ZRS. *Eur J Hum Genet* 18(6):733–736.
22. Gurnett CA, et al. (2007) Two novel point mutations in the long-range SHH enhancer in three families with triphalangeal thumb and preaxial polydactyly. *Am J Med Genet A* 143(1):27–32.
23. Semerci CN, et al. (2009) Homozygous feature of isolated triphalangeal thumb-preaxial polydactyly linked to 7q36: No phenotypic difference between homozygotes and heterozygotes. *Clin Genet* 76(1):85–90.
24. Wieczorek D, et al. (2010) A specific mutation in the distant sonic hedgehog (SHH) cis-regulator (ZRS) causes Werner mesomelic syndrome (WMS), while complete ZRS duplications underlie Haas type polysyndactyly and preaxial polydactyly (PPD) with or without triphalangeal thumb. *Hum Mutat* 31(1):81–89.
25. Wu L, et al. (2009) A ZRS duplication causes syndactyly type IV with tibial hypoplasia. *Am J Med Genet A* 149A(4):816–818.
26. Laurell T, et al. (2012) A novel 13 base pair insertion in the sonic hedgehog ZRS limb enhancer (ZRS/LMBR1) causes preaxial polydactyly with triphalangeal thumb. *Hum Mutat* 33(7):1063–1066.
27. Al-Qattan MM, Al Abdulkareem I, Al Haidan Y, Al Balwi M (2012) A novel mutation in the SHH long-range regulator (ZRS) is associated with preaxial polydactyly, triphalangeal thumb, and severe radial ray deficiency. *Am J Med Genet A* 158A(10):2610–2615.
28. Lettice LA, Hill AE, Devenney PS, Hill RE (2008) Point mutations in a distant sonic hedgehog cis-regulator generate a variable regulatory output responsible for preaxial polydactyly. *Hum Mol Genet* 17(7):978–985.
29. Al-Baradie R, et al. (2002) Duane radial ray syndrome (Okihiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. *Am J Hum Genet* 71(5):1195–1199.
30. Kohlhase J, et al. (2002) Okihiro syndrome is caused by SALL4 mutations. *Hum Mol Genet* 11(23):2979–2987.
31. Kohlhase J, Wischermann A, Reichenbach H, Froster U, Engel W (1998) Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome. *Nat Genet* 18(1):81–83.
32. Basson CT, et al. (1997) Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet* 15(1):30–35.
33. Basson CT, et al. (1999) Different TBX5 interactions in heart and limb defined by Holt-Oram syndrome mutations. *Proc Natl Acad Sci USA* 96(6):2919–2924.
34. Brassington AM, et al. (2003) Expressivity of Holt-Oram syndrome is not predicted by TBX5 genotype. *Am J Hum Genet* 73(1):74–85.
35. Fan C, Liu M, Wang Q (2003) Functional analysis of TBX5 missense mutations associated with Holt-Oram syndrome. *J Biol Chem* 278(10):8780–8785.
36. Li QY, et al. (1997) Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet* 15(1):21–29.
37. Barna M, Hawe N, Niswander L, Pandolfi PP (2000) Plzf regulates limb and axial skeletal patterning. *Nat Genet* 25(2):166–172.
38. Barna M, et al. (2002) Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev Cell* 3(4):499–510.
39. Fischer S, et al. (2008) Biallelic loss of function of the promyelocytic leukaemia zinc finger (PLZF) gene causes severe skeletal defects and genital hypoplasia. *J Med Genet* 45(11):731–737.
40. Jeannotte L, Lemieux M, Charron J, Poirier F, Robertson EJ (1993) Specification of axial identity in the mouse: Role of the Hoxa-5 (Hox1.3) gene. *Genes Dev* 7(11):2085–2096.
41. McIntyre DC, et al. (2007) Hox patterning of the vertebrate rib cage. *Development* 134(16):2981–2989.
42. Rancourt DE, Tsuzuki T, Capecchi MR (1995) Genetic interaction between hoxb-5 and hoxb-6 is revealed by nonallelic noncomplementation. *Genes Dev* 9(1):108–122.
43. Aubin J, Déry U, Lemieux M, Chailler P, Jeannotte L (2002) Stomach regional specification requires Hoxa5-driven mesenchymal-epithelial signaling. *Development* 129(17):4075–4087.
44. Zakany J, Zacchetti G, Duboule D (2007) Interactions between HOXD and Gli3 genes control the limb apical ectodermal ridge via Fgf10. *Dev Biol* 306(2):883–893.
45. Wada N, Kawakami Y, Nohno T (1999) Sonic hedgehog signaling during digit pattern duplication after application of recombinant protein and expressing cells. *Dev Growth Differ* 41(5):567–574.
46. Minguillon C, et al. (2012) Hox genes regulate the onset of Tbx5 expression in the forelimb. *Development* 139(17):3180–3188.
47. Kohlhase J, et al. (1999) Molecular analysis of SALL1 mutations in Townes-Brocks syndrome. *Am J Hum Genet* 64(2):435–445.
48. Bourgeois P, et al. (1998) The variable expressivity and incomplete penetrance of the twist-null heterozygous mouse phenotype resemble those of human Saethre-Chotzen syndrome. *Hum Mol Genet* 7(6):945–957.
49. Krawchuk D, et al. (2010) Twist1 activity thresholds define multiple functions in limb development. *Dev Biol* 347(1):133–146.
50. Zhang Z, et al. (2010) Preaxial polydactyly: interactions among ETV, TWIST1 and HAND2 control anterior-posterior patterning of the limb. *Development* 137(20):3417–3426.
51. Hasson P, Del Buono J, Logan MP (2007) Tbx5 is dispensable for forelimb outgrowth. *Development* 134(1):85–92.
52. Buaas FW, et al. (2004) Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* 36(6):647–652.
53. Itou J, et al. (2012) Islet1 regulates establishment of the posterior hindlimb field upstream of the Hand2-Shh morphoregulatory gene network in mouse embryos. *Development* 139(9):1620–1629.
54. Capellini TD, et al. (2006) Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression. *Development* 133(11):2263–2273.
55. Galli A, et al. (2010) Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. *PLoS Genet* 6(4):e1000901.
56. Zhang Z, Verheyden JM, Hassell JA, Sun X (2009) FGF-regulated Etv genes are essential for repressing Shh expression in mouse limb buds. *Dev Cell* 16(4):607–613.
57. Verheyden JM, Lewandoski M, Deng C, Harfe BD, Sun X (2005) Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning. *Development* 132(19):4235–4245.
58. Avantaggiato V, et al. (1995) Developmental analysis of murine Promyelocyte Leukemia Zinc Finger (PLZF) gene expression: Implications for the neuromeric model of the forebrain organization. *J Neurosci* 15(7 Pt 1):4927–4942.
59. Gong KQ, Yallowitz AR, Sun H, Dressler GR, Wellik DM (2007) A Hox-Eya-Pax complex regulates early kidney developmental gene expression. *Mol Cell Biol* 27(21):7661–7668.