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

Ben Xu[a,1], Steven M. Hrycaj[a], Daniel C. McIntyre[a,2], Nicholas C. Baker[a], Jun K. Takeuchi[b], Lucie Jeannotte[c], Zachary B. Gaber[d], Bennett G. Novitch[d], and Deneen M. Wellik[a,3]

[a]Department of Internal Medicine, Division of Molecular Medicine and Genetics, University of Michigan, Ann Arbor, MI 48109; [b]Cardiovascular Regeneration Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo 113-0032, Japan; [c]Centre de Recherche en Cancérologie de l'Université Laval, Centre Hospitalier Universitaire de Québec, Québec, Canada G1R 2J6; and [d]Department of Neurobiology, Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, David Geffen School of Medicine at UCLA, Los Angeles, CA 90095

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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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[1]Present address: Department of Human Genetics and Howard Hughes Medical Institute, University of Utah, Salt Lake City, UT 84112.

[2]Present address: Department of Biology, Duke University, Durham, NC 27708.

[3]To whom correspondence should be addressed. E-mail: dwellik@umich.edu.

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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

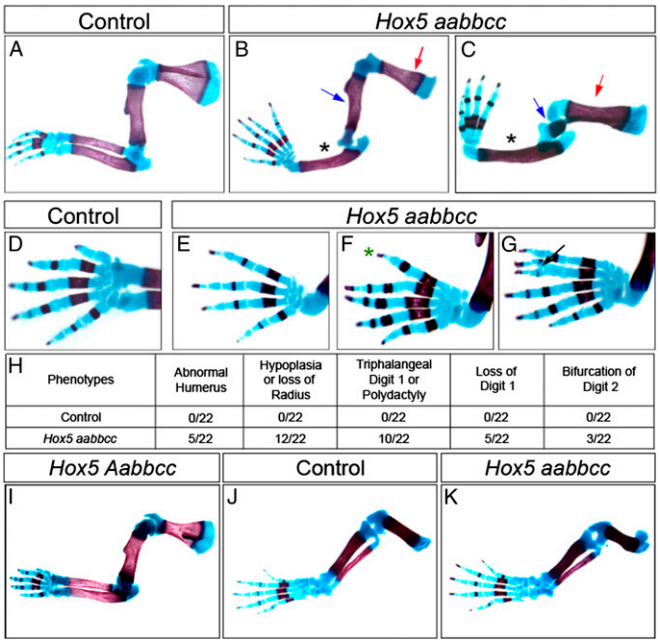

Fig. 1. Loss of function of *Hox5* paralogous genes results in anterior forelimb defects. (*A*–*G*) Skeletal analysis of control and *Hox5* triple-mutant forelimbs at E18.5. The scapula is reduced in *Hox5* triple mutants compared with controls, as observed for *Hoxa5* single mutants (*A*–*C*, red arrows). 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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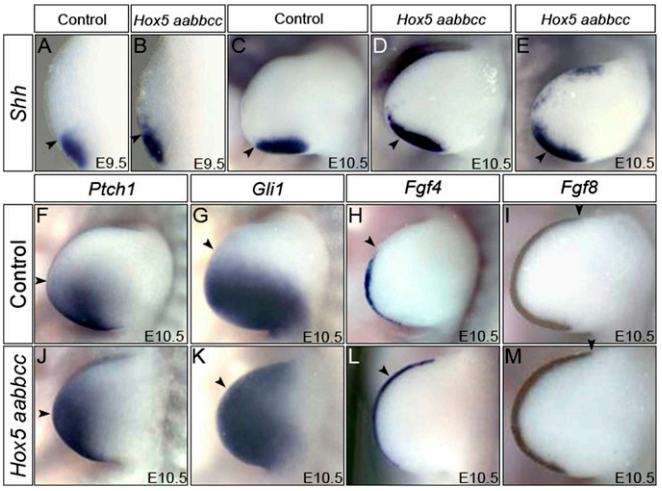


Fig. 2. *Shh* signaling is disrupted in *Hox5* mutant forelimbs. (*A–E*) *Shh* expression is anteriorized in *Hox5* mutant forelimbs. At E9.5, *Hox5* mutant forelimbs display slightly anteriorized *Shh* expression compared with controls (*A* and *B*). Anteriorization of *Shh* expression is observed by 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

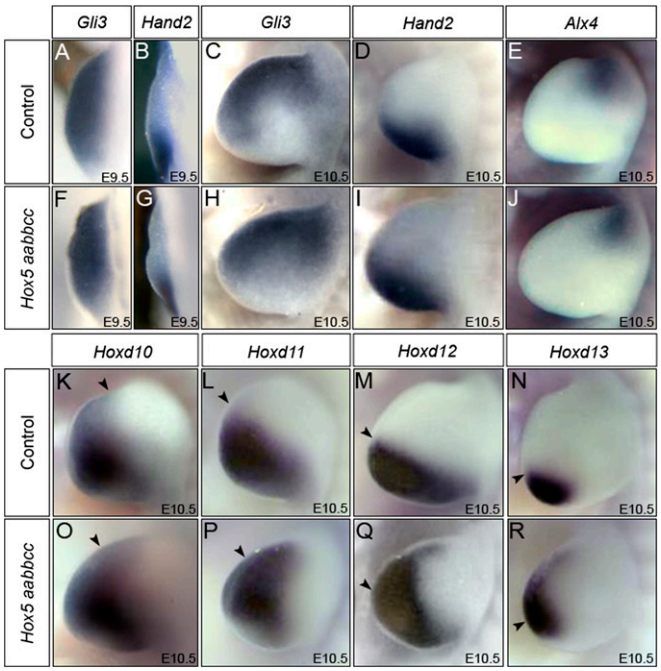


Fig. 3. Early limb patterning pathways are not disrupted, but posterior *HoxD* gene expression is anteriorized in *Hox5* mutants. (*A–J*) Early AP patterning factors are not disrupted in *Hox5* mutants. The expression of *Gli3* is not altered at E9.5 (*A* and *F*) or E10.5 (*C* and *H*). *Hand2* is expressed 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.

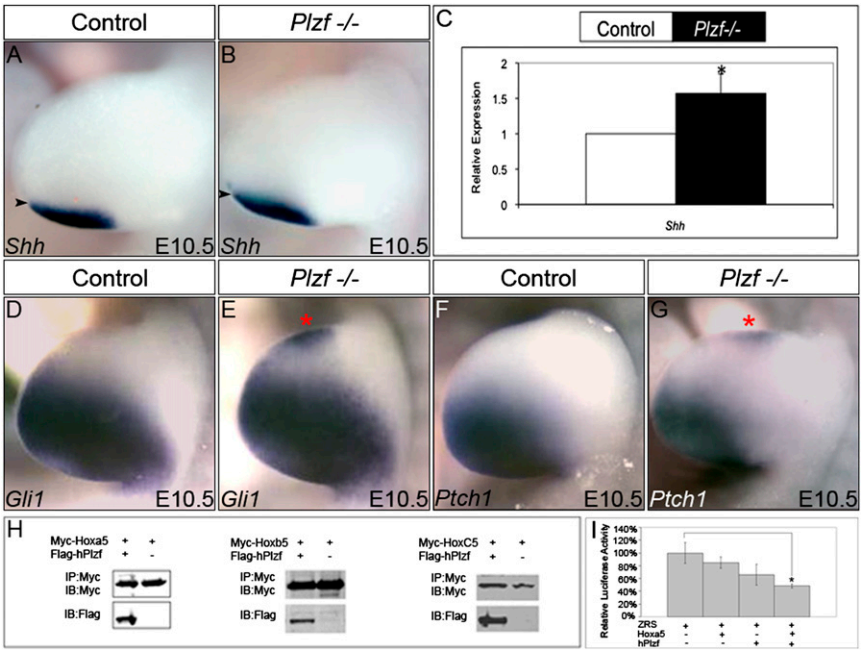
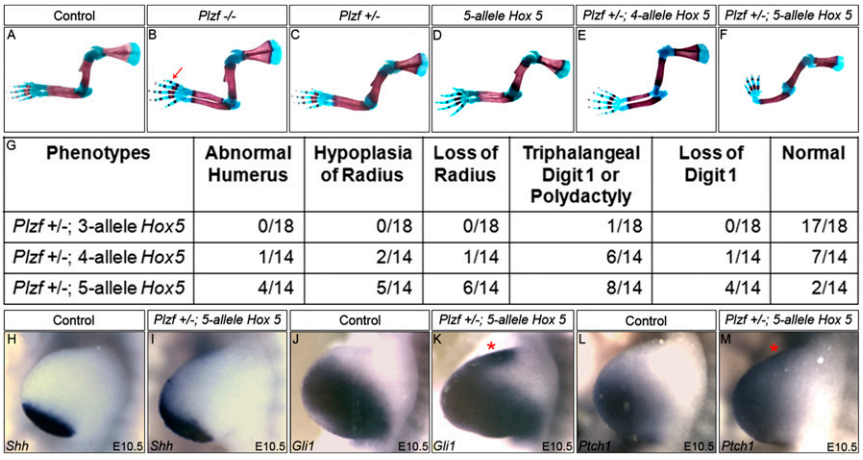


Fig. 4. Hox5 and Plzf cooperatively mediate repression of *Shh* expression via ZRS. (*A*–*G*) The *Shh* signaling pathway is disrupted in *Plzf* mutant forelimbs similar to that in *Hox5* mutant forelimbs. The expression of *Shh* is anteriorized in *Plzf* mutant forelimb buds compared with controls (*A* 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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