RESEARCH COMMUNICATION

Mutual genetic antagonism involving GLI3 and dHAND prepatterns the vertebrate limb bud mesenchyme prior to SHH signaling

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The bHLH transcription factor dHAND is required for establishment of SHH signaling by the limb bud organizer in posterior mesenchyme, a step crucial to development of vertebrate paired appendages. We show that the transcriptional repressor GLI3 restricts dHAND expression to posterior mesenchyme prior to activation of SHH signaling in mouse limb buds. dHAND, in turn, excludes anterior genes such as *Gli3* and *Alx4* from posterior mesenchyme. Furthermore, genetic interaction of GLI3 and dHAND directs establishment of the SHH/FGF signaling feedback loop by restricting the BMP antagonist GREMLIN posteriorly. These interactions polarize the nascent limb bud mesenchyme prior to SHH signaling.

Received October 25, 2001; revised version accepted December 28, 2001.

Development of paired appendages (limbs and fins) in vertebrates is controlled by a mesenchymal organizer located at the posterior limb bud margin (Johnson and Tabin 1997). Analysis of chicken and mouse limb bud development has shown that Sonic Hedgehog (SHH) is the morphogenetic signal expressed by the organizer (called polarizing region, or ZPA) that controls patterning of the distal limb bud (Chiang et al. 2001; Kraus et al. 2001). During progression of limb bud development, the polarizing region is maintained and propagated distally by an SHH/FGF signaling feedback loop. The SHH/FGF feedback loop is established in the posterior mesenchyme through localized activation of the BMP antagonist GREMLIN, which relays the SHH signal from responding mesenchymal cells to FGFs expressed by the posterior apical ectodermal ridge (AER; Zuniga et al. 1999; Sun et al. 2000). In turn, FGF signaling by the posterior AER maintains the SHH signaling polarizing region (Johnson and Tabin 1997). Interestingly, both GREMLIN in the posterior mesenchyme and FGFs in the

posterior AER are activated prior to SHH signaling (Zuniga et al. 1999), similarly to other posterior genes such as 5' *HoxD* genes (Kraus et al. 2001). These studies indicate that the nascent limb bud mesenchyme is already prepatterned (Chiang et al. 2001) and that the mesenchymal responsiveness to polarizing region signals is established prior to SHH activation. However, little is known about the mechanism acting upstream of SHH signaling to polarize the limb field and early limb bud. Several studies suggest a possible combinatorial involvement of *Hox* genes in positioning and polarizing the early limb field in response to retinoic acid (Lu et al. 1997). In particular, anterior ectopic expression of *Hoxb8* in forelimb buds of transgenic mouse embryos results in establishment of an ectopic polarizing region and mirror image duplication of distal limb structures (Charité et al. 1994). However, neither targeted inactivation of the *Hoxb8* gene (van den Akker et al. 1999) nor deletion of all *Hox8* paralogs in the mouse alters limb morphogenesis (van den Akker et al. 2001).

In contrast, analysis of the basic helix-loop-helix (bHLH) transcription factor dHAND (or HAND2) in zebrafish (Yelon et al. 2000), chicken (Fernandez-Teran et al. 2000), and mouse embryos (Srivastava et al. 1997; Charité et al. 2000) shows that dHAND is required for establishment of SHH signaling in both fin and limb buds. During embryonic development, dHAND is initially expressed throughout the flank mesenchyme and becomes restricted to the posterior mesenchyme during initiation of limb bud development (for review, see Cohn 2000). Therefore, identification of the gene(s) restricting dHAND to the posterior limb bud mesenchyme should provide insights into the genetic networks acting upstream of the polarizing region.

Disruption of the zinc finger protein GLI3 in the mouse results in establishment of a small anterior ectopic polarizing region and a polydactylous *Extratoes* (*Xt*) limb phenotype (Schimmang et al. 1992; Hui and Joyner 1993; Buscher et al. 1997). Normally, the full-length GLI3 zinc finger protein is processed to a transcriptional repressor (Wang et al. 2000), which participates in maintaining posterior restriction of SHH signaling during limb bud morphogenesis. SHH signaling by the polarizing region, in turn, inhibits the constitutive processing of GLI3, which results in formation of an anterior (high) to posterior (low) GLI3-R protein gradient (Wang et al. 2000). In addition to these reciprocal SHH-GLI3 interactions, genetic evidence for early GLI3 functions in establishing the polarizing region was also obtained (Zuniga and Zeller 1999). GLI3 is part of a larger number of genes whose disruption in the mouse results in establishment of an anterior SHH signaling center in limb buds and polydactyly (Masuya et al. 1995, 1997; Qu et al. 1997). Only a few of the disrupted genes have been identified, for example, the *paired* homeodomain transcription factor *Aristaless-like4* (*Alx4*). Disruption of the *Alx4* gene in the mouse causes *Strong's Luxoid* (*Lst*) polydactyly, and it has been proposed that ALX4, like GLI3, is part of the genetic mechanism that keeps SHH signaling restricted to the posterior limb bud mesenchyme (Qu et al. 1997; Takahashi et al. 1998).

In the present study, we establish that GLI3 is required to restrict dHAND expression to the posterior mesen-

[Key Words: *Alx4*; dHAND; *Gli3*; limb development; polarizing region; SHH signaling]

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Article and publication are at <http://www.genesdev.org/cgi/doi/10.1101/gad.219202>.

chyme during initiation of limb bud morphogenesis. We also show that up-regulation of *Alx4* expression depends on GLI3 function, thereby establishing that GLI3 acts initially upstream of ALX4. In turn, posterior dHAND function is required to keep both GLI3 and ALX4 restricted to anterior limb bud mesenchyme. Furthermore, dHAND positively regulates *Gremlin* expression and thereby differential responsiveness to SHH signaling, which, in turn, triggers establishment of the SHH/FGF feedback loop. Our studies reveal that the nascent limb bud mesenchyme is prepatterned by these genetic interactions prior to polarizing region signaling.

Results

GLI3 restricts dHAND to the posterior limb bud mesenchyme during initiation of limb bud outgrowth

dHAND is initially expressed throughout the lateral plate mesenchyme (Charité et al. 2000; Fernandez-Teran et al. 2000), but is restricted to the posterior mesenchyme during initiation of forelimb bud development by embryonic day 9.25 (E9.25; 22–23 somites; Fig. 1A). This correlates with activation of *Gli3* expression in the anterior limb bud mesenchyme (Fig. 1B; see also Masuya et al. 1997; Zuniga and Zeller 1999). Therefore, the distribution of dHAND was determined in forelimb buds of *Xt/Xt* homozygous embryos, which lack GLI3 function (Schimmang et al. 1992; Hui and Joyner 1993). In con-

Figure 1. *dHAND* expression is restricted to the posterior limb bud mesenchyme by *Gli3* repressor activity. (A) *dHAND* expression in the limb bud of a wild-type embryo (22–23 somites). *dHAND* expression is restricted to the posterior limb bud mesenchyme. (B) *Gli3* expression in the limb bud mesenchyme of a wild-type embryo (22–23 somites). (C) *dHAND* expression extends anteriorly in the limb bud of an *Xt/Xt* homozygous embryo (22–23 somites). (D,E) Expression of *dHAND* in limb buds of wild-type (D) and *Xt/Xt* (E) mutant embryos with 27 somites. *dHAND* expression is up-regulated in the posterior mesenchyme under the influence of SHH signaling (open arrowheads). All limb buds shown are forelimb buds. Arrowheads indicate the posterior margins of forelimb buds, with anterior to the top and posterior to the bottom.

trast to the rapid posterior restriction in wild-type limb buds (Fig. 1A, arrowheads), *dHAND* expression persists in anterior mesenchyme of *Gli3*-deficient limb buds (22–23 somites; Fig. 1C). During progression of limb bud development, *dHAND* remains expressed in the anterior limb bud mesenchyme of *Xt/Xt* embryos (27 somites; Fig. 1, cf. E and D). In *Gli3*-deficient limb buds, *dHAND* expression is lower in anterior than in posterior mesenchyme (Fig. 1E, open arrowhead) as *dHAND* expression is up-regulated under the influence of SHH signaling (Charité et al. 2000; Fernandez-Teran et al. 2000). *Shh* expression is first detected in posterior forelimb bud mesenchyme around E9.5 (25 somites; Masuya et al. 1997; data not shown). The observed polarized expression of *Gli3* (25 somites; Fig. 2A) and *dHAND* (Fig. 2B) is initially SHH-independent, as their complementary distribution is maintained in *Shh*-deficient limb buds (25 somites; Fig. 2C,D). During subsequent limb bud development, SHH signaling in posterior mesenchyme regulates *Gli3* expression negatively (Marigo et al. 1996) and *dHAND* positively (Charité et al. 2000; Fernandez-Teran et al. 2000). In older *Shh*-deficient limb buds, GLI3 is no longer excluded from posterior-most mesenchyme (Fig. 2E; see also Chiang et al. 2001), whereas the dHAND domain fails to extend distally (cf. Fig. 2F to 1D; see also Charité et al. 2000).

Expression of the transcription factor *Alx4* is activated in anterior limb bud mesenchyme with kinetics similar to *Gli3*. Therefore, the possibility that ALX4 could participate in restricting *dHAND* was also studied (Fig. 2G–L). In the absence of ALX4 function in *Lst/Lst* mouse embryos (25 somites), both posterior restriction of *dHAND* (Fig. 2, cf. G and H) and expression of *Gli3* (data not shown) are normal. In contrast, comparative analysis of *Alx4* in wild-type and *Xt/Xt* mutant limb buds shows that *Alx4* expression is initially lower in *Gli3*-deficient limb buds (25 somites; Fig. 2, cf. I and J). Subsequently, *Alx4* expression remains relatively restricted in *Xt/Xt* mutant limb buds (34 somites; Fig. 2, cf. K and L). The results presented in Figures 1 and 2 show that GLI3 represses *dHAND* and is necessary for positive regulation of *Alx4* in anterior mesenchyme.

dHAND keeps both GLI3 and ALX4 anteriorly restricted during onset of limb bud outgrowth

Previous analysis of *dHAND* mutant mouse embryos has established that morphogenesis of their limb buds is disrupted at an early stage. *dHAND*-deficient limb buds appear slightly smaller than their wild-type counterparts, and activation of both *5'HoxD* genes and *Shh* around E9.5 is disrupted (Charité et al. 2000; Yelon et al. 2000). To assess whether expression of anterior genes is also altered in *dHAND* mutant limb buds, the distributions of GLI3 and ALX4 were analyzed. Whereas *Gli3* is normally not expressed by posterior mesenchymal cells (Fig. 3A,C, arrowhead; see also Fig. 1B), it is expressed by posterior-most mesenchyme in *dHAND*-deficient forelimb buds from early stages onward (Fig. 3B,D, arrowheads). Similar to *Gli3*, the expression of *Alx4* is no longer restricted to the anterior-most mesenchyme (Fig. 3E), but is expanded posteriorly in *dHAND*-mutant limb buds (Fig. 3F). Taken together, these results show that both *Gli3* and *Alx4* are aberrantly expressed in posterior mesenchyme of *dHAND* mutant limb buds.

However, one possible explanation for the observed nonpolarized *Gli3* and *Alx4* expression could be the loss of posterior mesenchyme. *Hoxb8* is the earliest known marker for the posterior part of the forelimb field (Charité et al. 1994). By E9.5 (24–25 somites), weak *Hoxb8* expression is still observed in the posterior half of both wild-type and *dHAND* mutant forelimb buds (Fig. 3G,H), which shows that posterior mesenchyme is not lost in *dHAND* mutant limb buds at this stage. As *dHAND* is essential for survival of mesenchymal cells (Srivastava et al. 1997), TUNEL staining was used to assess a possible contribution of apoptosis to these early alterations of gene expression patterns. Analysis of serial sections revealed that no significant increase in cell death has occurred in the mesenchyme of both wild-type and *dHAND* mutant forelimb buds by E9.5 (24–25 somites; Fig. 3I,J), when expression of *Gli3* and *Alx4* has already been expanded posteriorly (Fig. 3B,D,F). However, the mesenchyme of *dHAND* mutant limb buds begins to undergo massive apoptosis by E9.75 (27 somites and older; data not shown). In summary, the results shown in Figure 3 establish that *dHAND* function is required to exclude *Gli3* and *Alx4* from the posterior limb bud mesenchyme.

The reciprocal GLI3–dHAND interactions participate in establishing differential responsiveness to future SHH signaling

The BMP antagonist GREMLIN participates in relaying the SHH signal from the mesenchyme to the AER during establishment and maintenance of the SHH/FGF feedback loop. Activation of *Gremlin* in the limb bud mesenchyme and *Fgf* expression in the posterior AER precedes SHH signaling (Zuniga et al. 1999). Interestingly, the expression of both *Gremlin* (Fig. 4B,D) and *Fgf4* (Fig. 4F) is anteriorly expanded in *Xt/Xt* limb buds (Fig. 4, cf. A,C,E to B,D,F). This anterior expansion has occurred by E9.75 (29–30 somites; Fig. 4B) and precedes detection of ectopic anterior SHH signaling in *Xt/Xt* limb buds by ~1 embryonic day (Buscher et al. 1997; Zuniga and Zeller 1999). In contrast, *Gremlin* expression is normal in *Alx4*-deficient limb buds during the same developmental period (data not shown).

One possible cause for this anterior expansion of *Gremlin* could be its up-

Figure 2. Complementary expression of *Gli3* and *dHAND* is independent of SHH signaling and *ALX4* function. (A,B) Complementary expression of *Gli3* (A) and *dHAND* (B) in contralateral limb buds of a wild-type embryo (25 somites). (C,D) Complementary expression of *Gli3* (C) and *dHAND* (D) in contralateral limb buds of an *Shh*-deficient embryo (25 somites). (E,F) Expression of *Gli3* (E) and *dHAND* (F) in contralateral limb buds of older *Shh*-deficient embryos (34 somites). (G,H) *dHAND* expression in wild-type (G) and *Lst/Lst* (H) mutant limb buds (25 somites). (I–L) Comparative analysis of *Alx4* expression in wild-type and *Xt/Xt* homozygous limb buds. (I) Wild-type (25 somites); (J) *Xt/Xt* homozygous (25 somites), arrowhead points to weak *Alx4* expression; (K) wild-type (34 somites); (L) *Xt/Xt* homozygous (34 somites). All limb buds shown are forelimb buds, with anterior to the top and posterior to the bottom.

regulation by ectopic *dHAND* function (Fig. 1). However, *dHAND* is not necessary for *Gremlin* activation, as

Figure 3. *dHAND* keeps *Gli3* and *Alx4* from being expressed by posterior limb bud mesenchyme. (A–D) *Gli3* expression in limb buds of wild-type (A,C) and *dHAND*-deficient (B,D) embryos (A,B: 22 somites; C,D: 24–25 somites). (E,F) *Alx4* expression in limb buds of wild-type (E) and *dHAND*-deficient (F) embryos (24 somites). (G,H) Expression of *Hoxb8* in limb buds contralateral to the ones shown in panels C and D. (G) Wild-type limb bud (24–25 somites); (H) *dHAND* mutant limb bud (24–25 somites). Arrowheads in panels A–H indicate posterior boundaries of expression domains. Asterisks indicate posterior edges of limb buds. (I,J) TUNEL analysis to detect apoptotic cells. (I) Wild-type limb bud (24 somites); (J) *dHAND* mutant limb bud (24 somites). White arrowhead points to apoptotic cells in a somite (Srivastava et al. 1997). All limb buds shown are forelimb buds, with anterior to the top and posterior to the bottom.

Figure 4. Genetic interaction of GLI3 and dHAND restricts GREMLIN-mediated competence to establish the SHH/FGF signaling feedback loop to the posterior limb bud mesenchyme. (A) *Gremlin* expression in a wild-type limb bud (29–30 somites). (B) *Gremlin* expression expands anteriorly in an *Xt/Xt* limb bud (29–30 somites). (C) *Gremlin* expression in a wild-type limb bud (37 somites). (D) *Gremlin* expression in an *Xt/Xt* limb bud (37 somites). (E,F) *Fgf4* expression in the limb buds contralateral to the ones shown in panels C and D. (E) Wild-type limb bud (37 somites); (F) *Xt/Xt* limb bud (37 somites). (G) Retroviral overexpression of *dHAND* in chicken limb buds results in similar up-regulation of *Gremlin* expression in the anterior mesenchyme (arrowhead) in all embryos analyzed ($n = 6$). All limb buds shown are forelimb buds, with anterior to the top and posterior to the bottom.

its expression is normal in *dHAND*-deficient limb buds around E9.5 (Charité et al. 2000; data not shown). To determine if ectopic dHAND can up-regulate *Gremlin* in anterior mesenchyme, the prospective wing bud region of chicken embryos was infected with a retrovirus encoding the dHAND protein. Such ectopic dHAND expression induces weak anterior SHH signaling and results in duplication of anterior digits in a fraction of all wing buds (for details, see Fernandez-Teran et al. 2000). In contrast, dHAND overexpression causes anterior up-regulation of *Gremlin* (Fig. 4G, arrowhead, embryonic stage 25) in all cases ($n = 6$). The *Gremlin* domain in such wing buds is similar to what is observed in *Gli3*-deficient limb buds (Fig. 4, cf. G and D).

Discussion

As summarized in Figure 5, the present study uncovers components of a regulatory mechanism that prepatterns the limb bud mesenchyme prior to SHH signaling by the polarizing region. *dHAND* is initially expressed by the lateral plate mesenchyme and becomes restricted to the posterior mesenchyme during initiation of limb bud

morphogenesis (Charité et al. 2000; Fernandez-Teran et al. 2000). Interestingly, this dynamic dHAND distribution largely parallels tissue competence to establish a polarizing region and activate SHH signaling. This competence is rather widespread but weak in flank mesenchyme prior to formation of limb buds (Tanaka et al. 2000). During initiation of limb bud outgrowth, both *dHAND* and the competence become restricted to and up-regulated in posterior mesenchyme. Indeed, genetic analysis of mouse and zebrafish embryos shows that dHAND is required to establish SHH signaling by the polarizing region in tetrapod limb buds (for review, see Cohn 2000). We now establish that GLI3-mediated transcriptional repression is crucial for restricting *dHAND* expression to the posterior mesenchyme (Fig. 5, pathway 1) concurrent with restriction of the competence to activate SHH signaling (Tanaka et al. 2000). Despite phenotypic and molecular similarities in the polydactylous limb phenotypes of *Gli3*- and *Alx4*-deficient mouse embryos (Qu et al. 1997; Takahashi et al. 1998), the posterior restriction of dHAND does not depend on ALX4 function. Rather, GLI3 function is required for positive regulation of *Alx4* expression, which places GLI3 genetically upstream of *Alx4* during initiation of limb bud morphogenesis (Fig. 5, pathway 2).

dHAND is genetically required to keep both *Gli3* and *Alx4* expression restricted to the anterior mesenchyme (Fig. 5, pathway 3). However, ectopic dHAND expression in chicken limb buds does not suffice to significantly down-regulate *Gli3* and/or *Alx4* in anterior mesenchyme (Fernandez-Teran et al. 2000). The repression of *Gli3* and *Alx4* may simply depend on formation of an active heterodimer between dHAND and another bHLH transcription factor (Firulli et al. 2000) expressed only in posterior mesenchyme. In addition, dHAND is required for transcriptional activation of several types of posterior patterning genes (Fig. 5, pathway 4), such as 5' *HoxD* genes, *Shh*, and *Bmp2* (Yelon et al. 2000). Interestingly, dHAND also regulates *Gremlin* positively, which, in turn, is part of the genetic cascades positioning the polarizing region and maintaining the SHH/FGF feedback

Figure 5. Reciprocal genetic repression between GLI3 and dHAND prepatterns the limb bud mesenchyme prior to activation of SHH signaling. (1) GLI3 repressor activity (GLI3-R) restricts expression of the bHLH transcription factor *dHAND* to the posterior mesenchyme during onset of limb bud morphogenesis. (2) GLI3-R participates in positive transcriptional regulation (dashed arrow) of another anterior transcription factor, *Alx4*. (3) dHAND is necessary to keep *Gli3* and *Alx4* expression restricted to the anterior mesenchyme. (4) In posterior mesenchyme, dHAND is necessary for activating expression of posterior genes, among them 5' *HoxD* genes, *Bmp2*, and *Shh* (for details and references, see text). These genetic interactions prepattern the limb bud mesenchyme independent of SHH signaling.

loop (Zuniga and Zeller 1999; Zuniga et al. 1999). Therefore, loss of posterior restriction of dHAND in *Gli3*-deficient limb buds is a likely cause of the anterior expansion of the 5' *HoxD* (Zuniga and Zeller 1999) and *Grem1* expression domains. This expansion long precedes establishment of a small anterior SHH signaling center. The analysis of *Shh*-deficient limb buds led Chiang et al. (2001) to conclude that the nascent limb field and early limb bud mesenchyme are prepatterned by an SHH-independent mechanism. The present study begins to uncover the molecular basis of this pre patterning mechanism and establishes that active cross-regulation between anterior and posterior mesenchyme is essential during initiation of limb bud outgrowth (Fig. 5). This pre patterning mechanism participates in determining posterior identity and positioning of the polarizing region and sets up differential mesenchymal responsiveness to future SHH signaling. As GLI3 functions first to restrict dHAND expression to posterior mesenchyme, establishment of the limb bud organizer seems triggered by anterior to posterior repression of activators rather than solely by posterior activation.

Materials and methods

Mouse strains and embryos

Gli3-deficient mouse embryos were obtained by intercrossing heterozygous mice carrying the *Xt^l* allele. The 3' part of the *Gli3* gene is deleted in the *Xt^l* allele, and mutant embryos were PCR-genotyped as described by Buscher et al. (1997). *Alx4*-deficient mouse embryos were obtained by intercrossing heterozygous mice carrying the *Lst^l* allele. *Lst^l* embryos were PCR-genotyped using a strategy based on the 16-bp deletion within the homeobox domain of the *Alx4* gene (Takahashi et al. 1998). *dHAND*-deficient embryos were obtained by intercrossing *dHAND* heterozygous mice and genotyped as described by Srivastava et al. (1997). *Shh*-deficient embryos were obtained by intercrossing heterozygous *Shh* mice and genotyped as described by St-Jacques et al. (1998).

Whole-mount in situ hybridization

Whole-mount in situ hybridization using digoxigenin-labeled RNA probes was performed as described by Haramis et al. (1995). The chicken *Grem1* probe was isolated by RT-PCR and its identity confirmed by DNA sequencing. Wild-type and mutant embryos were age-matched according to their somite numbers (variation ± 2 somites). Reproducibility of all results was ensured by analyzing several embryos ($n \geq 3$) in independent experiments.

Retroviral infection of chicken wing buds

RCAS-*dHAND* retroviral particles were injected into the presumptive wing field of chicken embryos (stage 12–14), and embryos were analyzed by in situ hybridization 3 d later (stage 25), as described in detail by Fernandez-Teran et al. (2000). Pathogen-free eggs (CRIFFA) were used for all studies, and embryos were staged according to Hamburger and Hamilton (1951).

Detection of apoptotic cells by TUNEL staining

Apoptotic cells were detected in situ by incorporating fluorescein-dUTP into fragmented DNA using terminal transferase according to the manufacturer's instructions (Roche Diagnostics).

Acknowledgments

We thank A. Beverdam (*Lst^l*) and D. Srivastava (*dHAND*) for making colony founders and probes for genotyping available to us. R. Dono, F. Meijlink, and A. Zuniga are thanked for reagents, advice, and many helpful discussions. We are grateful to S. Cohen, J. Deschamps, T. Durston, B. Scheres, and members of our groups for comments on the manuscript. This study is supported by NWO Grant 810.68.012 (R.Z.); the Faculty of Biology, Utrecht University (R.Z.); and the Spanish Ministry of Science Grants DGICYT-PM98-0151 and FIS 01/1219 (M.R.).

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Genes Dev. 2002, **16**:

Access the most recent version at doi:[10.1101/gad.219202](https://doi.org/10.1101/gad.219202)

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