An inversion involving the mouse Shh locus results in brachydactyly through dysregulation of Shh expression

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Short digits (Dsh) is a radiation-induced mouse mutant. Homozygous mice are characterized by multiple defects strongly resembling those resulting from Sonic hedgehog (Shh) inactivation. Heterozygous mice show a limb reduction phenotype with fusion and shortening of the proximal and middle phalanges in all digits, similar to human brachydactyly type A1, a condition caused by mutations in Indian hedgehog (IHH). We mapped Dsh to chromosome 5 in a region containing Shh and were able to demonstrate an inversion comprising 11.7 Mb. The distal breakpoint is 13.298 kb upstream of Shh, separating the coding sequence from several putative regulatory elements identified by interspecies comparison. The inversion results in almost complete downregulation of Shh expression during E9.5–E12.5, explaining the homozygous phenotype. At E13.5 and E14.5, however, Shh is upregulated in the phalangeal anlagen of Dsh/+ mice, at a time point and in a region where WT Shh is never expressed. The dysregulation of Shh expression causes the local upregulation of hedgehog target genes such as Gli1-3, patched, and Pthlh, as well as the downregulation of Ihh and Gdf5. This results in shortening of the digits through an arrest of chondrocyte differentiation and the disruption of joint development.

Introduction

Formation of the vertebrate appendicular skeleton involves a number of sequential phases resulting in the formation of the various tissues of the limb in a proximodistal sequence (reviewed in ref. 1). After the initial patterning phase, mesenchymal cells aggregate and differentiate into chondrocytes, forming the cartilaginous anlagen of the future skeleton. Mesenchymal condensations resemble the future skeleton in shape and size. In many instances, though, more than 1 bone or cartilage may arise from a single condensation. This is particularly evident in the limbs. The digital rays, consisting of the metacarpals/tarsals and 3 phalanges (proximal, middle, and distal) in digits II–V and 2 phalanges in digit I, are thought to be formed from prechondrogenic condensations that appear spatially continuous and subsequently segment into individual skeletal elements (1). The process of segmentation begins with a change in morphology of cells at the sites of joint formation. The cells become flattened, are more densely packed, lose their chondrogenic characteristics, and change their extracellular matrix composition (2, 3). In the chick, this so-called interzone develops into a 3-layered structure, with the central region undergoing apoptosis, resulting in the formation of the joint cavity (4).

A range of human skeletal dysplasias and mouse models indicate that joint formation is closely linked to proper chondrocyte differentiation and vice versa. Mutations in the genes for Indian hedgehog (IHH) (5), growth and differentiation factor 5 (GDF5) (6), BMP-receptor 1B (BMPR1B) (7), and the receptor tyrosine kinase ROR2 (8, 9) have been shown to cause distinct subtypes of brachydactyly, a group of inherited disorders of the hands characterized by shortening of the digits together with abnormal phalangeal joint formation (10). Ihh, a secreted signaling peptide from the hedgehog (Hh) family, is expressed in prehypertrophic chondrocytes. It activates Pthlh, which is expressed in periarticular cartilage, and inhibits the conversion of proliferating and prehypertrophic chondrocytes into hypertrophic chondrocytes, establishing a feedback loop between Ihh and Pthlh (11). The role of Ihh during cartilage differentiation and joint formation is underscored by the fact that mutations in IHH cause human brachydactyly type A1 (12). The cross-talk between Ihh and Pthlh is not only important for the regulation of chondrocyte differentiation in the growth plate but also essential for proper joint formation.

Another central molecule involved in the pathogenesis of brachydactyly is Gdf5, a signaling molecule from the TGF-β superfamily expressed in the perichondrium and joint interzones of the developing appendicular skeleton (13). Lack of Gdf5 function in the mouse mutant brachypodism leads to shortening of limbs, brachydactyly, and loss of phalangeal joints (14), similar to the human acromesomelic dysplasias of the Grebe (15) and Hunter Thompson types (16) that are also caused by mutations in GDF5. Gdf5 signals through its receptor Bmpr1b. Mutations in BMPR1B lead to brachydactyly type A2 (17) and a subset of acromesomelic dysplasia (18). Mutations in ROR2, a receptor tyrosine kinase, result in brachydactyly type B, a condition characterized by loss of distal

Nonstandard abbreviations used: BAC, bacterial artificial chromosome; CNE, conserved noncoding element; Dsh, short digits; Dsh+/−, Dsh heterozygote; EST, expressed-sequence tag; Hh, hedgehog; P1, proximal phalanx; P2, middle phalanx; P3, distal phalanx.

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thought to result in a global downregulation of gene activity lead far away (up 1 Mb) from the gene. Such mutations are generally cent to the gene (19). In these cases, the breakpoint can be located cis to the gene (19). Expression by disrupting the promoter and transcribed exons from as inversions and translocations can interfere with normal gene logical states. For example, chromosomal rearrangements such nisms that can disrupt normal gene function leading to patho...phalanges and symphalangism (9). The role of Ror2 in joint formation is less clear, but recent evidence points to a modulation of the Gdf5/BmpR1b pathway by Ror2 (18).

The above-mentioned disease-associated mutations alter the protein coding sequence of the gene in some way and thus cause the phenotype. However, there are potentially many other mecha...sions of Shh expression in the limbs during morphogenesis of the phalanges, resulting in short digits and a brachydactyly phenotype. The Dsh mutant represents an as yet undescribed example of the temporal and spatial misregulation of gene expression due to a chromosomal rearrangement. The developmental pathology associated with this misexpression extends our understanding of the developmental pathology of brachydactyly.

**Results**

Dsh/Dsh phenotype. The Dsh/Dsh phenotype is characterized by multiple internal and skeletal defects (Figure 1A) (20). Dsh/Dsh embrios have a severe defect in midline patterning, and the musculature is virtually absent (21). Skeletal malformations are characterized by a complete lack of craniofacial bone, but the persistence of some cartilaginous tissues was evident in the craniofacial region. Furthermore, absence of vertebral bodies, dorsal ribs, and distal limb structures was observed. Scapula and humerus articulate, but the single bone that replaces radius and ulna is fused to the humerus. In the hindlimb, the femur is present and articulates to a tibia, which is truncated shortly after the joint. The distal limb structures (autopod) are absent in the hindlimbs and represented by a single, digit-like element in the forelimbs. The Dsh/Dsh and Shh+/- phenotypes are very similar (22). However, E17.5 Dsh/Dsh mice are larger in size, and the proboscis in Dsh/Dsh tends to be larger than in Shh+/- mice. To test whether Dsh and Shh are allelic, Shh+/- mice were crossed with Dsh heterozygote (Dsh/+ ) mice and the double heterozygotes were compared with Dsh/Dsh and Shh+/- mice. Dsh/+; Shh+/- mice revealed a phenotype that was nearly identical to that of Shh+/- and Dsh/Dsh mice, with the full spectrum of midline defects, internal anomalies, and limb malformations, indicating that Dsh and Shh are allelic and do not complement (Figure 1A).

In order to examine the expression of Shh in Dsh/Dsh embryos, quantitative RT-PCR with total RNA from E10.5–E13.5 WT, Dsh/Dsh, Dsh/+ and Shh+/- embryos were performed. The results show an almost complete loss of Shh expression in Dsh/Dsh embryos until E12.5. At E12.5 and E13.5, however, residual Shh expression is detectable. Quantification of Shh expression in comparison to the corresponding WT at E12.5 and E13.5 shows a reduction to 10% and 27%, respectively (Figure 1B). At least some of the residual expression at E12.5 and E13.5 was shown by in situ hybridization to be due to ectopic expression of Shh in the proboscis of Dsh/Dsh embryos (data not shown).

**Mapping and mutation analysis.** Our initial mapping showed linkage to proximal chromosome 5 within a region containing the Shh gene. In order to screen for mutations in the Shh cDNA and nearby regulatory elements, we PCR-amplified and sequenced the floor plate enhancer 10 kb upstream of Shh and the 14-kb genomic region comprising exons 1–3, the promoter region, and the brain enhancer (Figure 2D) using cDNA and genomic DNA from Dsh/Dsh, C57BL10, C3H, and 101 mouse strains. No mutation was found. Several 101 strain polymorphisms were detected within the region, demonstrating that the original mutation had been induced in the 101-strain chromosome of the (101 × C3H) F1; male that had been irradiated. The critical region was further refined using a mapping strategy with a Dsh/C57BL10 BALBc intercross. Twenty-five recombinants for D5Mit226 were identified, reducing the mapping interval at the centromeric margin. Six recombinant mice were identified for marker D5SN13 at the telomeric margin still including the Shh gene (Figure 2A).
The large interval and the low number of recombinants were suggestive of a chromosomal rearrangement in the interval. To analyze such rearrangements with fluorescence in situ hybridization (FISH), 2 bacterial artificial chromosome (BAC) contigs covering both ends of the mapping interval were identified (chori, ensemble) and hybridized on interphase and metaphase spreads of spleen lymphocytes of Dsh/+ mice (Figure 2C). Analysis of the hybridization experiments revealed a split signal for BAC RP23-94F17 at 26.731374 Mb, 13.298 kb upstream of \( \text{Shh} \) and includes the floorplate enhancer. BLAST analysis of expression of flanking genes, \( \text{Shh} \), \( \text{Dsh} \) and \( \text{Lmbr1} \) residing at the centromeric breakpoint and \( \text{Rnf32} \) and \( \text{Sema3C} \) at the telomeric end showed a split signal and the order of telomeric and centromeric BACs is changed to blue/green and red/green (BAC 1,2,3), thus demonstrating the inversion. In the WT 3 colors are visible, in the order of red and green/blue (from centromere to telomere, BAC 1,2,3). In the Dsh mutant, BAC RP23-94F17 (green, BAC 1) shows a split signal and the order of telomeric and centromeric BACs is changed to blue/green and red/green (BAC 1,2,3), thus demonstrating the inversion. (D) Genomic region of the proximal and distal breakpoints. Genes are indicated by boxes, the direction of transcription is marked by arrow. FPE, floor plate enhancer; BE, brain enhancer; Pr, promoter; ZRS, regulatory element.

Identification of conserved elements. In order to identify conserved noncoding elements (CNEs) around the distal breakpoint, sequence comparisons were made with MultiPIPMaker and mVISTA programs using genomic DNA sequence from mouse, human, chick, and \( \text{Fugu rubripes} \). This analysis revealed 4 (CNE1–CNE4) cross-species conserved elements distal of the breakpoint, making it highly likely that expression of these genes contributes to the \( \text{Dsh}/\text{Dsh} \) or \( \text{Dsh}+/+ \) phenotype.

The Dsh/+ limb phenotype is a model for human brachydactyly. \( \text{Dsh}/+ \) mice have short digits because of a fusion of the first and second phalanges of digits II–V and a shortened proximal phalanx of digit I (Figure 4). The carpal/tarsal bones and the remainder of the appendicular skeleton show no changes. Beginning at E14.5, chondrogenesis is delayed in the phalanges and metacarpals/tarsals of \( \text{Dsh}/+ \) mice, as demonstrated by a lack of alcian blue staining (Figure 4C). By E15.5, the proximal (P1), middle (P2), and distal (P3) phalanges have formed in the WT and are separated by joints. At this time point, a P1/P2 fusion bone is present in \( \text{Dsh}/+ \) mice that is not completely separated from the metacarpal by a joint. P3 is formed regularly. The P1 of digit I is split. In limbs of 4-week-old \( \text{Dsh}/+ \) mice, digits II–V are severely shortened due to a fused P1/P2, and mild shortening of the metacarpals is present (Figure 4A). The P3 and the distal interphalangeal joint appear normal. In digit IV the metacarpophalangeal joint is fused. Fore- and hindlimbs are localized the second breakpoint at 15.006770 Mb. Details of the genomic organization around the breakpoints are shown in Figure 2D.

To analyze the effect of the inversion on the level of expression of flanking genes, \( \text{Hgf}, \text{Estg3581}, \text{Speer4f}, \text{and Sema3C} \) residing at the centromeric breakpoint and \( \text{Rnf32} \) and \( \text{Lmbr1} \) at the telomeric end were tested with quantitative RT-PCR in E10.5–E13.5 WT and \( \text{Dsh}/\text{Dsh} \) embryos. No difference in expression was observed (data not shown), making it unlikely that expression of these genes contributes to the \( \text{Dsh}/\text{Dsh} \) or \( \text{Dsh}+/+ \) phenotype.

Figure 2
\( \text{Dsh} \) is caused by an inversion involving the \( \text{Shh} \) gene. (A) Mapping of \( \text{Dsh} \) shows linkage to mouse chromosome 5 in a region containing \( \text{Shh} \). A schematic of proximal chromosome 5 is shown on left. Enlarge-ment of the indicated region shows the linkage interval with the marker tested, the \( \text{Shh} \) and \( \text{Lmbr1} \) genes, and the number of recombinant mice observed per 100 meioses (Recombl/100 meioses). Note repression of recombination in the inversion interval. Mb, mega-base; cM, centi Morgan. (B) Southern blot analysis using a probe spanning the distal breakpoint showing aberrant bands in \( \text{Dsh}/\text{Dsh} \) mice. (C) Fluorescence in situ hybridization with BACs from mouse chromosome 5 that flank the inversion breakpoints. In the WT 3 colors are visible, in the order of red and green/blue (from centromere to telomere, BAC 1,2,3). In the Dsh mutant, BAC RP23-94F17 (green, BAC 1) shows a split signal and the order of telomeric and centromeric BACs is changed to blue/green and red/green (BAC 1,2,3), thus demonstrating the inversion. (D) Genomic region of the proximal and distal breakpoints. Genes are indicated by boxes, the direction of transcription is marked by arrow. FPE, floor plate enhancer; BE, brain enhancer; Pr, promoter; ZRS, regulatory element.
affected to the same extent. Therefore, the Dsh/+ phenotype is very similar to human brachydactyly type A1.

H&E-stained limb sections from E13.5–E16.5 mice were prepared for a more detailed analysis of chondrogenesis and joint formation (Figure 4D). In WT mice, differentiation of chondrocytes and formation of a joint interzone between the phalanges is starting at E13.5 and is clearly visible at E14.5. In the P1 and P2 skeletal elements of Dsh/+ mice, chondrocytes remain undifferentiated, do not align in columns, and do not hypertrophy. Proliferation in Dsh/+ limbs was analyzed with BrdU incorporation at E13.5–E16.5. At E14.5, BrdU-labeled cells are seen in prehypertrophic chondrocytes, the perichondrium, and the joint interzones. In Dsh/+ digits, BrdU labeling is similar to WT in the perichondrium, but is reduced in nondifferentiating chondrocytes and absent where joints should form (Figure 4E). TUNEL immunodetection in digits of WT mice showed apoptosis in the interdigital mesenchyme, the central layer of the 3-layered joint interzone. In Dsh/+ mice, no apoptotic signals were detected, reflecting disturbed joint cavitation (Figure 4F). In addition, apoptosis in the interdigital web was reduced. Taken together, these findings indicate that the Dsh/+ limb reduction phenotype is a result of delayed chondrogenesis, abnormal chondrocyte differentiation, reduced proliferation, and disruption of joint formation. The large number of proliferating cells in the area of the developing joints argues for the presence of a specific growth center in this region in the WT. The absence of this growth region is likely to contribute to the shortening of the individual phalangeal elements.

**Ssh expression in Dsh/+ limbs.** To investigate Ssh expression in Dsh/+ limbs, we performed a series of in situ hybridization experiments using a Ssh-specific probe. Whole-mount in situ hybridization confirmed a normal expression pattern until E11.5 in the posterior mesenchyme (see Figure 6A). In situ hybridization of E13.5 and E14.5 on Dsh/+ limbs showed ectopic expression of Ssh in the cartilaginous anlagen of the developing digits (Figure 5A). In accordance with the Dsh/+ phenotype, expression of Ssh was observed only in the phalanges and not in the metacarpals or other structures of the developing limb. Hh signal is known to induce the expression of Ptihb over a distance in the joint region (11). Most likely as a result of ectopic Ssh expression, Ptihb is ectopically expressed throughout the phalangeal cartilage anlage beginning at E13.5. Quantitative RT-PCR shows upregulation of Ptihb beginning at E13.5 (Figure 5F). Ihb expression in Dsh/+ mice showed a regular pattern in the metacarpal/tarsal anlagen and at the tip of the digits. However, we never observed Ihb expression in those elements that ultimately develop into the proximal and middle phalanges, the major site of the Dsh/+ phenotype (Figure 5C). To determine the specificity of the Shh probe and to rule out cross-hybridization with Ihh, WT and Dsh/+ E13.5 and E14.5 limbs were hybridized with Shh- and Ihb-specific probes. No staining was observed in WT limbs using the Shh-specific probe (Figure 5A), indicating that the signal observed in Dsh/+ mice was specific. In contrast, hybridization with the Ihb-specific probe gave a strong signal in the developing metacarpal and phalangeal anlagen in WT limbs, whereas Dsh/+ limbs showed a signal only in the metacarpal but not the phalangeal anlagen (Figure 5C). Thus, the areas of Shh expression correlate with the region where Ptihb is upregulated and Ihb is downregulated.

To determine whether Ssh mRNA observed by in situ hybridization is translated, we performed immunohistochemistry with an antibody recognizing Hh proteins. We observed distinct staining of prehypertrophic chondrocytes in the WT metacarpals and phalanges, corresponding to Ihh expression. In the Dsh mutant, we observed regular staining in the metacarpals, corresponding to Ihh. However, in the phalanges, diffuse staining within the chondrocytes and in the flanking perichondrium was observed, corresponding to Shh protein (Figure 5C). These results are in good agreement with the in situ hybridization analysis.

**Ssh expression was further analyzed using quantitative RT-PCR (Figure 5D).** At E10.5 and E11.5, Shh expression was reduced to approximately 50% of WT levels, as expected for a heterozygous regulatory mutation. Similar results were obtained in Shh−/+ mice (data not shown). Ssh expression is strongly downregulated in WT limbs at E12.5 and is practically switched off thereafter. A similar degree of downregulation was observed in the Dsh mutant at E12.5. However, in contrast to the WT, Shh expression was strongly upregulated at E13.5 and E14.5 in the mutant. Thus, the Dsh mutant behaves like a heterozygous loss-of-function mutation during the regular stages of Shh expression (i.e., E9.5–E11.5). Thereafter, Shh is turned off in the WT but strongly upregulated in the mutant.

**Developmental analysis of Dsh/+ limbs.** To evaluate the effect of Shh misexpression, we analyzed the expression of Shh targets and other genes involved in digital patterning and initial chondrogenesis including Ptc, Gli1-3, Fgf4, Fgf8, Bmp2, Bmp4, Bmp7, Gremlin, Fornin, Hoxd11-13, Gdf5, Nog, Col2a1, and Col10a1. In accordance with the normal expression of Shh at E10.5–E12.5 (Figure 6A), all tested genes displayed a regular expression pattern during the developmental stage at E10.5–E12.5 (Figure 6B). Concomitant with the upregulation of Shh at E13.5, we observed major differences in gene expression between WT and Dsh/+ mice (Figure 6C).

Analysis of Hh downstream targets such as the Hh receptor Patched (Ptc) and Gli1 demonstrated ectopic expression throughout the putative joint region in Dsh/+ mice. Gli2 and Gli3, which have repressor activity and antagonize Gli1 and Shh function, are expressed in the joint and periartricular region (23). In Dsh/+ limbs, Gli2 is expressed in the immature chondrocytes of the P1/P2 fusion
phalax, and Gli3 expression is shifted from the joint to the peri-
chondrium and the interdigital mesenchyme in the mutant.

Gdf5 is a signaling molecule from the TGF-β superfamily that
is expressed in early cartilage condensations, the perichondrium,
and the joint interzones of the developing appendicular skeleton.
In E13.5 digits of Dsh+/− mice, Gdf5 is found in the perichondrium
but does not show the characteristic striped pattern in the joint
interzone. At E14.5, Gdf5 is expressed in a longitudinal stripe in the
region where the putative joint would be established.

The differentiation of chondrocytes is delayed in Dsh+/− digits,
as shown by reduced collagen type II expression in early stages
and absence of collagen type X expression, normally expressed in
hypertrophic chondrocytes, in later stages.

Discussion
Proteins of the Hh family are key mediators of many fundamental
processes in embryonic development (for review see ref. 24). Shh
is the most prominent member of the Hh family of signaling mol-
eules. It is involved in a variety of processes during development,
malformation, and cancer. Loss of both Shh copies in mice leads
to a severe midline defect and a variety of internal and skeletal
malformations (22). In humans, heterozygous loss-of-function
mutations in SHH result in holoprosencephaly, characterized by
medial cleft lip and palate, single maxillary incisor, frontal CNS
lobe fusion, and midfacial defects, including a spectrum rang-
ing from hypotelorism to cyclopia (25, 26). Hh ligands elicit their
effects by antagonizing the activity of their receptor patched (ptc),
which binds a third protein, smoothened (Smo), a G protein–cou-
pled receptor. The cubitus interruptus orthologs Gli1, Gli2, and
Gli3 mediate the transcriptional control by Hh signaling in verte-
brates, with Gli1 being an activator of Hh target gene activation,
whereas Gli2 and Gli3 are considered to be repressors. Partial loss
of this repressor activity underlies the various human and murine
polydactylies that are caused by mutations in the Shh-Gli signal
transduction pathway (for review see ref. 27).

Dsh is a regulatory Shh mutation. Homozygous Dsh/Dsh embryos
display a phenotype highly similar to the Shh+/− phenotype. However,
in contrast to Shh+/− mice, the Dsh+/− mutant is characterized by short
digits, a skull defect, and other anomalies (20). Using a positional
cloning approach, we demonstrate that Dsh is caused by an intrachro-
mosomal inversion that comprises 11.7 Mb. In accordance with the
presence of an inversion, we observed a suppression of recombination
in proximity to the breakpoints, a phenomenon thought to be due to
the formation of inversion loops during meiosis causing unstable
chromosomes. The inversion is likely to be caused by the initial irra-
diation experiment, because ionizing radiation frequently results in
DNA double-strand breaks and genomic rearrangements (28).

One breakpoint of the Dsh inversion is 13 kb upstream of the
Shh gene. Hence, the genomic rearrangement leaves the exons,
the promoter, and 2 enhancers (29) intact. In humans, transloca-
tions 15–265 kb 5′ of the SHH promoter have been described that
result in a holoprosencephaly phenotype similar to that observed
in individuals with mutations in SHH (26). These findings can be
interpreted as a long-range position effect due to position effect

Figure 4
The Dsh+/− limb phenotype. (A) Aliza-
rin red–stained skeletal preparations
of 4-week-old WT and Dsh+/− fore-
limb digits. MC, metacarpals. Digits
I–V are shown. In Dsh+/− mice, the
P1 and P2 elements are fused (P1/2,
arrows) and severely reduced in size.
In digit IV, the metacarpal-phalangeal
joint is also fused. MC, metacarpals.
(B) Hindlimb of WT and Dsh+/− adult
mouse showing severe reduction in
digit length. (C) Alcian blue staining
of E15.5 WT and Dsh+/− limbs. Note
lack of the middle phalanx anlage
in all digits and persistent staining
between the developing metacarpal
and P1, indicating delayed or absent
joint formation (arrow). (D) H&E
staining of E14.5 WT and Dsh+/−
digits. WT shows the beginning of
joint formation (arrow). In contrast,
Dsh+/− shows lack of joint interzone
formation and undifferentiated chon-
drocytes in the region of prospective
phalanges (arrow). (E) BrdU stain-
ing of E14.5 WT and Dsh+/− autopod
(top) and digits (bottom) showing
reduced proliferation in the region of
joint interzones (arrow). Magnifica-
tion, ×18 (A), ×7 (B), ×40 (C), ×50
(E), ×100 (D and F).
be expected to lead to reduction or absence of the promoter/transcription unit from such elements can be expected to lead to reduction or absence of the promoter/transcription unit from such elements. The separation of controlled genes and were shown to confer position-independent, site-specific expression explains the severe midline defect and the associated variegation, a phenomenon describing the variable but heritable inhibition of gene expression due to an altered chromatin environment through a chromosomal rearrangement (19). However, the presence of multiple conserved sites upstream of the Shh gene, as shown by our interspecies sequence comparisons (Figure 3), is highly suggestive of the presence of locus control regions. Such sites have been found to be associated with a number of tightly clustered genes and were shown to confer position-independent, copy number–dependent expression of a linked gene. The separation of the promoter/transcription unit from such elements can be expected to lead to reduction or absence of Shh transcription. As shown by quantitative RT-PCR, this is the case, at least during the early stages of development. The almost complete loss of Shh expression explains the severe midline defect and the associated malformations in Dsh/Dsh mice that are indistinguishable from the Shh/– phenotype. This suggested that Dsh and Shh are allelic, a hypothesis supported by our complementation experiments. However, the situation is more complex, as demonstrated by the upregulation of Shh expression after E13.5. The Dsh inversion results not only in the downregulation of Shh expression but also in a dysregulation.

Experiments performed in transgenic zebrafish and mice indicate that Shh expression is controlled by multiple transcriptional regulators (29, 30). One Shh enhancer element was identified in intron 5 of the flanking Lmbr1 gene located approximately 1 Mb away from Shh. In the Sqq mouse mutant, this element (designated ZRS) is disrupted (31), resulting in the ectopic expression of Shh at the anterior margin of the limb in addition to its normal posterior expression domain, and, consequently, in a polydactyly phenotype. Point mutations in this element segregate with polydactyly in 4 unrelated postaxial polydactyly families (31) and in the mouse mutant hemimelic extraroots (Hx) (32). Using a genetic test with the Sqq, Hx, and Shh alleles, it was elegantly shown that the ZRS element is a cis-acting regulator (31, 33). Against the background of these findings, a 6-kb deletion including exon 4 and the surrounding intronic sequence of the of the human Lmbr1 homolog C2ORF7 in achondroplasia, a malformation syndrome with a limb truncation phenotype similar to that seen in the Shh/– mice (34), may be interpreted as loss of another Shh enhancer element, possibly belonging to an enhancer repressor complex. In addition to the regulatory elements discussed, other sites must exist that control the ZPA-specific expression of Shh, as shown in the mouse mutant replicated anterior zeugopod (raz) (35) and the chick mutant oligozeugodactyly (ozd) (36). Similar to Dsh, raz is caused by an inversion involving the Shh locus. The breakpoints, however, are not well defined and appear to reside 12 Mb distal of Lmbr1 and 3 Mb proxim-
mal of Shh. Raz and ozd result in the downregulation of Shh expression in the limb and a successive loss of Shh target gene expression, suggesting that they interfere with a ZPA-specific enhancer. Such elements may reside within the conserved sequences identified in this study that are located between the Dsh breakpoint and the Lmbr1 gene. The different phenotypes in acheiropodia, Dsh, and Ssq suggest that activating and/or repressing properties may be located near the ZRS. In addition to removing an enhancer element from the genomic context necessary for proper expression of Shh, the Dsh inversion could also eliminate an element involved in silencing Shh. The loss of this putative silencer would then result in ectopic Shh expression. In addition, Lmbr1 may be involved in Shh activation, as Lmbr1+/− mice with no phenotype show loss of posterior digits when crossed with mice carrying a deletion of chromosome 5 including Shh (37). Retroviral overexpression of Lmbr1 in chick limbs, however, has no effect (38).

Other possibilities to consider are the disruption of a second gene involved in the regulation of Shh expression in trans or the translocation of a new promoter element before the Shh gene resulting in the misexpression of Shh via this element. A detailed analysis of the genes and expressed-sequence tags (ESTs) located in the vicinity of the centromeric breakpoint revealed no gene spanning a breakpoint, and no differences in the expression of nearby located genes were observed, making the hypothesis of a second gene unlikely.

Figure 6
Developmental analysis of Dsh/+ limbs. Expression of marker genes relevant for digit development was performed using whole-mount (A and B) and section (C) in situ hybridization. (A) Expression of Shh in the posterior limb region and downregulation at E12.5 showing no difference between WT and Dsh/+ . At E12.5 (B), we find normal patterns of expression for all genes tested. At E13.5 and E14.5 (C), the majority of genes show an abnormal pattern. Note misexpression of Hh receptor patched (ptc) and downstream effectors Gli1–Gli3 (arrows). Gdf5 first shows lack of expression in the developing joints (E13.5) and is then (E14.5) expressed in a more longitudinal fashion in the area of the future metacarpophalangeal joints (arrow). Col2a1 (Collagen type II a1; Col2) is expressed throughout the phalangeal anlagen but is less pronounced in the distal regions of Dsh/+ digits. Lack of Col10a1 (Collagen type X a1; Col10) expression in digit anlagen demonstrates delay in chondrocyte differentiation in Dsh/+ . Magnification, ×50 (A–C).
Furthermore, our complementation experiments strongly argue against this possibility. None of the tested ESTs or genes showed an expression pattern compatible with the ectopic expression of Shh in Dsh/+ mice, rendering unlikely the possibility that the inversion puts Shh under the control of a new regulatory element that might alter its normal expression pattern. However, we cannot completely rule out this possibility, because the chromosomal region around the proximal breakpoint is not fully characterized.

Dsh is a model for abnormal joint formation. We propose that the brachydactyly phenotype in Dsh is causated by the reactivation of Shh expression in the perichondrium and cartilage of the digital anlagen. The misexpression of Shh results in a upregulation of Shh targets and, consequently, in the disruption of the Ihh-Pthlh feedback loop and other signaling pathways involved in joint development, such as the BMP/Gdf5 pathway (Figure 7). Owing to the late onset of misexpression, the phenotype is temporally and spatially restricted to the development of the proximal and middle phalanges and thus does not show a typical Shh-related phenotype such as polydactyly or oligodactyly. Consistent with this hypothesis, we can show normal expression of molecular markers for limb patterning and cartilage development until E12.5 in the Dsh/+ digits.

The major players known to be involved in the organogenesis of phalanges and the interphalangeal joints are Ihh, the Ihh-Pthlh regulatory loop, and Gdf5. As shown by overexpression in the chick and gene inactivation experiments in mice, Ihh regulates chondrocyte differentiation in the growth plate, joint formation, and osteoblast differentiation in endochondral bones (11, 12). Mutations in IHH cause the human conditions acrocapitofemoral dysplasia (39) and brachydactyly type A1 (5). At E13.5, Ihh expression condenses in the middle of the metacarpal anlagen and starts to be visible first in the P1 and then in the P2. In Dsh/+ mice the metacarpal expression domain is maintained, but expression in the proximal and middle phalanges is absent. In spite of the missing Ihh expression, Hh downstream genes such as Gli1 and ptc are highly expressed, suggestive of an Hh signal not detected by our Ihh-specific probe. Searching for such a signal, we identified Shh expression in the phalangeal anlagen of Dsh/+ digits. Shh, Ihh, and the third member of the Hh family, Desert hedgehog (Dhh), are highly homologous, use the same receptors and signal transduction pathways, and are able to replace one another in appropriate in vitro and in vivo models (40). It is therefore conceivable that Shh elicits the same response, possibly with variable potency, as Ihh, when expressed in cartilaginous tissue. We propose that the ectopic expression of Pthlh as well as the loss of Ihh expression in the phalangeal anlagen of Dsh/+ mice is induced by Shh overexpression.

In the WT mouse, Pthlh is induced at a distance from the Hh signal, enabling the formation of a growth plate architecture with graded levels of chondrocyte differentiation. This pattern is completely disrupted in Dsh/+ phalanges, resulting in the absence of a regular growth plate and the persistence of chondrocytes in an undifferentiated state. Consistent with our hypothesis is the finding that overexpression of Pthlh in chondrocytes using a collagen type II promoter leads to a delay in mineralization and chondrocyte maturation (41). Likewise, addition of the active NH2 terminal fragment of Shh in limb cultures and overexpression of Ihh by retroviral gene transfer in chick limbs both delay hypertrophy of chondrocytes by upregulation of Pthlh at the periarticular joint region (11, 42). Consequently, the addition of active Shh has no effect in Pthlh–/– mice (11), and Ihh–/– mice express no Pthlh (12). Furthermore, overexpression of Ihh (Ihh) in chick embryos results in the downregulation of endogenous Ihh, probably by the upregulation of Pthlh (11). Similar results were obtained in the mouse when overexpressing Ihh or Shh in chondrocytes (43, 44), supporting our observation that Ihh is downregulated in areas of Shh misexpression.

These findings indicate that the perturbation of the Ihh-Pthlh feedback loop in Dsh/+ is responsible for the delay in chondrocyte differentiation and hypertrophy, resulting in shortening of the P1 and P2. In addition to the delay in chondrocyte differentiation, Pthlh overexpression and disruption of the physical distance between Ihh and Pthlh expression domains can be expected to interfere with the segmentation process. Gdf5 has a key role in joint formation, as demonstrated by the absence of interphalangeal joints in the brachypodism mouse, which lacks functional Gdf5 (14). The lack of Gdf3 expression at the appropriate sites is expected to contribute strongly to the Dsh/+ phenotype.

The Hh antagonists Gli2 and Gli3 also show a major expression domain in the developing joint, whereas Gli1 is spared from the joint and expressed around the cartilage anlage. Gli1 shows an identical ectopic expression as Shh in Dsh/+ mice, underscoring its role as transcriptional activator and primary mediator of Hh function. Conversely, Gli2 and Gli3 have repressor activity and antagonize Hh and Gli1 function (23). In Dsh/+ limbs, they have lost their normal pattern and are expressed throughout the autopod. Because the Gli genes are direct downstream targets of Hh signaling, their abnormal expression is likely to be induced by the ectopic Shh expression observed in Dsh/+ limbs. Misexpression of Gli1, Gli2, Gli3, and Gdf5 either directly or indirectly disrupts the intricate process necessary for joint formation and patterning and thus further contributes to the process. Inactivation of Ihh, as in the Ihh knockout mouse, as well as Gdf5 in the brachypodism mouse leads to the loss of joints. Ihh is not expressed in digits of brachypodism mice, and Gdf5 is upregulated in the peri-
chondrium of \(Sbh^{--}\) mice (data not shown), suggesting that Ihh and Gdf5 may regulate each other’s expression. The hypothesis presented here is supported by the finding that overexpression of \(Sbh\) in chick limbs also results in the loss of joints (45), as in \(Dhb^{+/+}\) digits.

The developmental analysis of digits and joints in the \(Dhb^{+/+}\) mutant indicates that the formation of phalangeal elements and joints mutually depend on each other. The interplay of Gdf5, Gli2, Gli1, and Pthlh in the joint space with Ihh expressed in the middle of the anlage is likely to influence the spacing of the joints and the size of the phalangeal elements (Figure 7). In this model, the developing joint region serves as a secondary signaling center that coordinates the size and number of cartilage elements by regulating proliferation, cell recruitment, chondrocyte differentiation, and, finally, joint formation. Human and mouse phenotypes with hypoplasia/aplasia of phalanges and/or abnormal interphalangeal joint formation are likely to be caused by a disruption of this process. Furthermore, this study shows that chromosomal rearrangements can result in unexpected misregulation of gene expression during development, a mechanism that may occur more frequently than previously thought.

Methods

**Mice.** \(Dhb\) is an autosomal semidominant mutation that was initially found in one of the progeny of a (101 × C3H) \(F_1\) hybrid male that had been exposed to 600 Röntgen (R) + 600 R of \(96\) R/min x-ray irradiation, with an 8-week interval between exposures (20). The mutant was subsequently kept on the C3H/101 background and was then crossed onto a C57BL10 background. Genotype analysis was performed by PCR amplification of extraembryonic membranes or tails using the DSM572 microsatellite marker, which is polymorphic for 101 and C57BL10 backgrounds and is located within the \(Dhb\) linkage interval on mouse chromosome 5. PCR was performed in 50-μl reactions containing 1.5 mM MgCl₂ and 2 pmol/μl of each primer. PCR-amplified DNA products of sizes 132 bp for C57BL10 and 140 bp for 101 were visualized on 4% ethidium bromide–stained agarose gels. \(Sbh^{+/+}\) mice served for complementation assay with \(Dhb^{+/-}\) and were genotyped as described elsewhere (22). This study was performed according to the guidelines of the ethics committee of the Landesamt für Arbeitsschutz, Gesundheitsschutz und technische Sicherheit, Berlin, Germany.

**Linkage analysis and fine mapping.** Linkage of \(Dhb\) was established by crossing \(Dhb^{+/-}\) females with \(Mus\) \(castaneus\) males. \(F_1\) mice that showed the \(Dhb^{+/+}\) phenotype were intercrossed. One hundred and nine mice from the \(F_2\) generation with a \(Dhb^{+/+}\) phenotype served for linkage analysis using a genome-wide set of microsatellite markers. In a second step, a fine-mapping analysis was performed by crossing \(Dhb^{+/-}\) females with BALB/c males. This cross generated 950 mice with a \(Dhb^{+/+}\) phenotype that were tested using publicly available polymorphic markers and markers generated using the Tandem Repeats Finder program version 2.02 (http://tandem.bu.edu/). Labeling of the markers was performed with a single-reaction nested PCR method using a fluorescent dye–labeled M13-21 primer (46) and analyzed on the ABI 3100 Prism Genetic Analyzer (Applied Biosystems).

**Identification of breakpoints.** Three-color FISH was performed on metaphase chromosomes and nuclei of \(Dhb^{+/+}\) splenocytes in accordance with standard procedures. BACs were labeled with Biotin-16-DUTP, Digoxigenin-11-DUTP (Roche Diagnostics Corp.), or Tamra-DUTP (Applied Biosystems) (red). Biotin-16-DUTP was visualized with FITC (green) and Digoxigenin-11-DUTP with Cy5 (far red). Fluorescent signals were documented with an epifluorescence microscope (Leica DMRXA) equipped with a cooled charge-coupled device camera (Photometrics) and band-pass filters (Chroma Technology Corp.). The QFISH-software (Leica Microsystems) was used for the analysis of the FISH results. Southern blot analysis was performed according to standard protocols with BamHI-, EcoRV-, PstI-, EcoRI-, and HindIII-digested DNA and analyzed using a PhosphorImager system (Molecular Dynamics). The genomic region of the \(Sbh\) gene was amplified with long-range PCR (Roche Diagnostics Corp.) from the mouse strains \(Dhb/Dhb\), C57BL10, C3H, and 101; cloned into TopoXL Kit (Invitrogen Corp.); sequenced; and analyzed on an ABI 3100 sequencer (Applied Biosystems). Genomic walking was performed using GenomeWalker Kit (Clontech). All sequences were annotated according to ENSEMBL Mouse genome v3.32.1.

**Skeletal preparations, in situ hybridization, and histological analysis.** Timed matings were performed for the analysis of the mutant phenotype, and skeletal preparations were prepared as described elsewhere (47). Histology, whole-mount and \(3^{3P}\) in situ hybridization was performed as previously reported (48). \(Dhb^{+/-}\) and WT embryos at E10.5, E11.5, and E12.5 served for whole-mount hybridizations. Limbs of E13.5–E14.5 mice were used for section in situ hybridization. The following riboprobes were used: \(Alx4\), \(Bmp2\), \(Bmp4\), \(Bmp7\), \(Col2a1\), \(DHand\), \(Fgf4\), \(Fgf8\), Gdf5, Gli1, Gli2, Gli3, Hoxa11, Hoxd11, Hoxd12, Hoxd13, Ihh, Noggin, PtC, Pthlh, Pthrl, and \(Sbh\) (47–49).

\(BrdU\) incorporation into DNA strands was used to analyze proliferation of chondrocytes as described previously (48). Apoptosis was analyzed with the TUNEL assay using ApopTag kit (Intergen) in accordance with the manufacturer’s protocol. Immunohistochemistry was performed with an anti-Shh antibody (ShhhAb80) as described previously (50).

**Quantitative RT-PCR.** Quantitative PCR was performed from RNA extracted from limb buds and whole embryos. Limb buds of E10.5–E14.5 WT and \(Dhb^{+/-}\) embryos were removed, collected in RNA-later (Qiagen) and genotyped. For RNA extraction, a minimum of 10 limbs were pooled, minced with an Ultra-Turrax T8, and extracted using Trizol (Invitrogen Corp.). For whole embryos, E10.5–E13.5 \(Dhb/Dhb\), \(Dhb^{+/-}\) and \(Sbh^{--}\) embryos were collected and extracted with Trizol. One microgram of RNA of each sample was transcribed to cDNA (Taqman Reverse Transcription Reagents; Applied Biosystems). PCR was performed in a 30-μl reaction containing 10 μl 1:10 diluted cDNA or standard, 5 μl primer mix (7.5 pmol of each primer), and 15 μl SYBR green PCR master mix (Applied Biosystems). PCR was performed in triplicate on an ABI Prism 7900 HT Sequence Detection System. Relative quantification of gene expression was performed using the standard curve method as described by the manufacturer (User Bulletin #2, 10/2001; Applied Biosystems).

**Comparative sequence analysis.** Sequence alignments between the species mouse, human, chicken, and \(F.\) \(rubripes\) were made with the MultiPIPMaker and mVISTA programs (51, 52) through their websites (http://pipmaker. bx.psu.edu/cgi-bin/multipipmaker and http://www-gsd.lbl.gov/vista/). Sequences of the various genomes used for alignments were obtained from ENSEMBL (http://www.ensembl.org).

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