The molecular basis of Pallister–Hall associated polydactyly

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Mutations in GLI3 manifest in several distinct clinical phenotypes including Greig cephalopolysyndactyly syndrome and Pallister–Hall syndrome (PHS). GLI3 belongs to the GLI family of transcription factors that mediates extracellular Sonic hedgehog (SHH) signals. In the absence of SHH signals, GLI3 is processed to form a transcriptional repressor termed GLI3R. During early limb development, the regulation of GLI3 processing by SHH is decisive in determining the correct number and identity of digits. Analyses of mouse embryos have produced evidence that elevated levels of GLI3R reduce the number of developing digits. Remarkably, PHS causative mutations are predicted to produce a truncated protein similar to the endogenous GLI3R. Nevertheless, polydactyly is frequently observed in PHS patients and it even represents a criterion for the clinical diagnosis of PHS. In order to detect the underlying cause of this obvious discrepancy, we made use of the Gli3-699 mouse mutant, which represents the mouse model of PHS. We show that the mutant murine allele gives rise to a truncated version of GLI3 that mimicks both the processed GLI3R isoform and the proposed pathogenic GLI3PHS protein. We analyzed how the mutant GLI3 protein interferes with the anteroposterior patterning of early limb development, whereas processes that are associated with the outgrowth of the limb bud remain remarkably unimpaired. The presented findings help to understand the previously enigmatic emergence of Pallister–Hall associated polydactyly and thus add to the understanding of the pathogenic mode of the action of GLI3PHS.

INTRODUCTION

Pallister–Hall syndrome (PHS) was first described in 1980 (1). It is a rare, autosomal-dominant malformation syndrome with a wide spectrum of severity including neonatal lethality (1). PHS has been shown to be caused by mutations in the middle third of the GLI3 gene, which have been predicted to result in a truncated GLI3 protein (2,3). The clinical diagnostic criteria for PHS require the presence of a hypothalamic hamartoma and insertional polydactyly (4). However, as yet, the emergence of polydactyly seems to conflict considerably with the genetic cause of the PHS.

The GLI family of transcription factors (GLI1-3) comprises the mediators of extracellular Sonic hedgehog (SHH) signals (5,6). Analyses of chick and mouse embryos have revealed that, in the absence of SHH signaling, the normal full-length GLI3 protein (GLI3-FL) is processed to a shortened isoform, generally termed GLI3 repressor (GLI3R) (7,8). GLI3R can then enter the nucleus and act as a repressor of SHH target genes.

In the developing limb bud, expression of SHH in the posterior mesenchyme creates an anteroposterior (AP) GLI3-FL:GLI3R gradient, which determines digit number and identity (8). The total lack of GLI3 function in the Gli3-deficient mouse mutant extra-toes (XtJ) leads to pronounced polydactyly associated with the loss of digit identities (9). Complete deprivation of SHH function in the Shh-/- mutant mouse results in severe skeletal deficiencies distal to the stylopod–zeugopod junction (elbow/knee joints); all zeugopod and autopod elements are either missing, fused or lack normal identity, except for a single digit 1 in the hind limb (8,10).

One very remarkable observation had a considerable impact on the conceptions concerning GLI3R function in limb development. It has been shown that the limbs of Gli3-/- Shh-/- embryos are virtually indistinguishable from polydactylous Gli3-/- limbs (8,11). These results implied that the most
of GLI3 is extensively phenocopied by the polydactyly of PHS patients (2, 3). Furthermore, the pleiotropic disease pattern of PHS is extensively phenocopied by the polydactyly of PHS patients (2, 3). However, given the polydactyly of PHS patients, the concept of the pathogenic mechanism is clearly inconsistent with the findings from murine embryonic development. Remarkably, as yet, no experimental study has ever analyzed this surprising incoherence of human and murine phenotypes.

In order to address this task, we made use of the mouse model for PHS. The Gli3<sup>Δ699</sup> mutant carries a targeted mutation 3′ of the sequences encoding the DNA-binding domain (five zinc fingers) at the Gli3 locus (13). Detailed analysis of the mutant transcript predicted a truncated GLI3 protein whose molecular size is within the range that has been suggested for the endogenously produced GLI3R isoform (7). In addition, the truncating nature and the position of the Gli3<sup>Δ699</sup> mutation also correspond to mutations identified in PHS patients (2, 3). Furthermore, the pleiotropic disease pattern of PHS is extensively phenocopied by the Gli3<sup>Δ699</sup> mutant (13). Here, we further examined the anatomical and molecular consequences of the truncated GLI3 protein in the context of limb development. Our findings reveal broad congruence of the mutant phenotypes of mouse and man. Moreover, our analysis of the transregulative properties of GLI3<sup>Δ699</sup> adds 21 codons in frame (13). We investigated the resulting protein with respect to its molecular size and expression level. Mutant and endogenous protein lysates were extracted from Gli3<sup>+/+</sup>, Gli3<sup>Δ699/Δ699</sup> and Gli3<sup>Δ699/Δ699</sup> embryos (E10.5). Both the full-length form of 190 kDa and the processed form of 83 kDa were detected by a GLI3 specific antibody in wild-type embryos (Fig. 1, left lane). In homozygous mutant embryos, neither the 190 kDa nor the 83 kDa protein was detectable (Fig. 1, right lane). Instead, solely the Gli3<sup>Δ699</sup> protein of about 88 kDa was identified. The quantity of Gli3<sup>Δ699</sup> in homozygous embryos was comparable with the totalized amount of wt GLI3 proteins within the same embryo (Fig. 1, central lane). Thus, the mutation in Gli3<sup>Δ699</sup> mutant mice indeed produces a truncated GLI3 protein, whose physical properties are similar to both the processed form of wild-type GLI3 (GLI3R) and the predicted aberrant GLI3-PHS protein (2, 3, 7).

RESULTS

The GLI3<sup>Δ699</sup> protein is similar to the processed form of GLI3 both in abundance and in molecular size

Previous analysis of the Gli3<sup>Δ699</sup> transcript revealed that the mutation terminates the Gli3 sequence at codon 699 and adds 21 codons in frame (13). We investigated the resulting protein with respect to its molecular size and expression level. Mutant and endogenous protein lysates were extracted from Gli3<sup>Δ699</sup> homozygous embryos (E10.5). Both the full-length form of 190 kDa and the processed form of 83 kDa were detected by a GLI3 specific antibody in wild-type embryos (Fig. 1, left lane). In homozygous mutant embryos, neither the 190 kDa nor the 83 kDa protein was detectable (Fig. 1, right lane). Instead, solely the Gli3<sup>Δ699</sup> protein of about 88 kDa was identified. The quantity of Gli3<sup>Δ699</sup> in homozygous embryos was comparable with the total quantity of full-length and processed GLI3 isoforms in wild-type embryos. This was further supported by the analysis of protein lysates isolated from homozygous embryos. There too did the quantity of Gli3<sup>Δ699</sup> match the totalized amount of wt GLI3 proteins within the same embryo (Fig. 1, central lane).

Digit number and identity are affected in Gli3<sup>Δ699/Δ699</sup> embryos

In this article, we will focus on skeletal development at the autopod level, even though the Gli3<sup>Δ699</sup> mutation also does affect more proximal parts of the limb skeleton, which had been discussed previously (13). A readily noticeable characteristic of the Gli3<sup>Δ699/Δ699</sup> forelimb was its clearly reduced size compared with heterozygous and wild-type littermates. The digits seemed to be shorter and the handplate in general appeared to be smaller. Bone and cartilage stainings revealed a disturbed ossification at E18.5 (Fig. 2). In the wrist of Gli3<sup>Δ699/Δ699</sup> mutants, the number of cartilage elements was reduced, showing considerable fusion of the carpals. The morphology of the metacarpus was also considerably compromised especially towards the posterior margin (Fig. 2B). The digits of Gli3<sup>Δ699/Δ699</sup> embryos
displayed a reduced level of dismorphology compared with the more proximal parts since the majority of interphalangeal segmentations seemed to be complete. The majority of Gli3ΔD699 embryos were polydactyous (7/10 limbs). Typically, they developed a single extra digit (6/10) but we also observed one heptadactylous fore limb (1/10) (Fig. 2B, data not shown). At a considerably lower frequency (2/10), we noted a reduction of the normal digit number (Fig. 2C).

Apart from affecting digit number, the Gli3ΔD699 mutation also affected digit identity, i.e. AP patterning. The normal pentadactylylimb of the mouse exhibits an obvious asymmetry along the AP axis. The anterior-most digit, digit 1, is different from its more posterior counterparts in that it is shorter and it only contains two phalanges, whereas the other digits possess three. The morphology of the mutant digits, however, indicated abnormal AP patterning. Even though most polydactylylimbs of Gli3ΔD699 embryos developed merely a single supernumerary digit, there were usually three pre-axial digits, which were biphalangeal and shorter than the central digits (Fig. 2B). Even in the oligodactylylimbs, we found an increased share of digits of anterior-most character as judged by morphometric criteria such as the number of phalanges and overall digit length (Fig. 2C). Moreover, Gli3ΔD699 embryos displayed malformations of the posterior digits. In all cases of polydactylylimbs, the two postaxial digits ematated from a single metacarpal; often there was only one phalan-geal joint in the posterior-most digit (Fig. 2B). Remarkably, the digits of oligodactylylimbs displayed a continuing increase in length from anterior to posterior (Fig. 2C). Since, normally, digit length reaches its climax at the central position (Fig. 2A), the silhouette of the oligodactylymutant fore limbs implied the absence of the most posterior digits (Fig. 2C).

As in the fore limb, the skeletal elements in the hind limb were highly dysplastic (Fig. 2E and F). At the tarsal level, we observed various degrees of fusion between the elements. In the midfoot region, the two posterior metatarsals were fused in 50% of cases (Fig. 2E). In general, the mutant hind limbs were pentadactylylimbs (8/10) (Fig. 2E). One hind limb had developed an additional sixth digit (data not shown), whereas the other hind limb consisted of only four digits (Fig. 2F). Without exception, the hind limb digits consisted of only two phalanges (10/10) (Fig. 2E and F). This was not due to the absence of a tip structure because the distal-most phalanx was clearly recognizable. Thus, the exclusive expression of Gli3ΔD699 affects digit number and identity in fore and hind limbs. On the basis of the criterion that only the anterior-most digit in the wild-type is biphalangeal, most of the digits in Gli3ΔD699 embryos exhibit an anterior character.

Zone of polarizing activity formation and apical ectodermal ridge activity were not affected in Gli3ΔD699 limbs

Probably the most striking feature of Gli3ΔD699 limbs is the emergence of supernumerary digits. Current hypotheses would predict that excessive GLI3R function should produce a nearly digitless phenotype that is similar to what is seen in Shh−/− mutants. Consequently, we were interested in examining the effects of Gli3ΔD699 on such gene expression that is associated with the outgrowth of the limb bud. The two main signaling centers in the early limb bud are the apical ectodermal ridge (AER) and the zone of polarizing activity (ZPA). The reciprocal positive interaction of AER and ZPA is pivotal in regulating limb bud outgrowth and digit number (14). One of the
The expression pattern of fore (A–I) and hind limb buds (J–O) at E11.0–11.5. (A–C) The expression of \(Gli3\) was unaffected. (M–O) While \(Fgf8\) was most prominent in the hind limb bud (Fig. 3E). However, in \(Shh\) mutant, expression was found to be expanded anteriorly in the AER of \(Gli3^{−/−}\) embryos (Fig. 3K) (21). However, expression analysis of the limbs of \(Gli3^{−/−}\) embryos revealed no differences to wild-type limbs (Fig. 3L). Since marginal differences in \(Fgf8\) activity could not be excluded by this analysis, we investigated the expression of \(Gremlin\), a key player in the maintenance of the SHH/FGF feedback loop (22). In line with the observed increase in \(Fgf8\) expression, we found an anterior expansion of \(Gremlin\) expression in \(Gli3^{−/−}\) limb buds (Fig. 3N). Expression of \(Gremlin\) in \(Gli3^{−/−}\) limbs was unaltered (Fig. 3O), which indicated that the AER/ZPA feedback loop is uncompromised in \(Gli3^{−/−}\) limbs. Therefore, a reduction of AER activity can be excluded as the cause of an increased share of biphalangeal digits in \(Gli3^{−/−}\) embryos.

**Analysis of AP-specific gene activity**

Since the phenotype of \(Gli3^{−/−}\) limbs strongly suggested alterations in the AP axis specification, we monitored molecular changes specific for the anterior and posterior polarity.

The transcription factor \(ALX4\) is expressed in the anterior mesenchyme of the early limb bud (Fig. 4A). Loss of \(ALX4\) function is associated with ectopic anterior ZPA formation and inappropriate AP patterning (23). In the \(Gli3^{−/−}\) mutant, \(Alx4\) expression is markedly reduced, whereas it is normal in \(Gli3^{−/−}\) embryos (Fig. 4B and C). \(Pax9\) has been reported to act downstream of \(Gli3\) in autopod development and to contribute to the patterning of the anterior skeletogenic mesenchyme (24,25). Targeted loss of \(Pax9\) results in pre-axial digit duplication (24). \(Pax9\) expression in the murine limb is first observed at E11.5 (26). We investigated \(Pax9\) as a late anterior marker at E12.5. In wild-type embryos, \(Pax9\) was expressed in a very prominent anterior domain and in a much weaker posterior domain (Fig. 4D). In \(Gli3^{−/−}\) limb buds, posterior \(Pax9\) expression persisted, whereas the anterior domain was strongly downregulated (Fig. 4E). In contrast, in \(Gli3^{−/−}\) limb buds, \(Pax9\) expression was normal, if not slightly upregulated anteriorly (Fig. 4F).

Recent evidence suggested that \(Hoxd\) genes participate in prepatternig events. The early posterior restriction of \(Hoxd\) gene products is decisive in setting up an AP prepattern (Fig. 4G), which determines the subsequent localized activation of \(Shh\) expression (27). Upon loss of \(Gli3\), \(Hoxd12\) factors known to interfere with the AER/ZPA feedback loop is GLI3 itself (15). At E11.0, \(Gli3\) is expressed most strongly in the anterior and the central distal regions of the limb bud (Fig. 3A). Expression from the \(Gli3\) null allele showed a remarkably similar pattern (Fig. 3B) despite the loss of early prepattern by reciprocal repression of \(Gli3\) and \(dHand\) (16). Likewise, exclusive expression from the \(Gli3^{Δ699}\) allele did not result in a noticeable change in expression pattern, revealing a similar horseshoe shape as in the normal limb bud (Fig. 3C).

\(Shh\) expression marks the ZPA, a group of mesodermal cells located at the posterior border of the normal limb bud (17). Similar to many polydactylous mouse mutants, \(Gli3^{−/−}\) embryos display ectopic anterior \(Shh\) expression, which was most prominent in the hind limb bud (Fig. 3E). However, in \(Gli3^{Δ699}\) embryos, \(Shh\) expression was normal (Fig. 3F). The expression of the Hedgehog receptor \(Ptc\) serves as an indicator of active SHH signaling (18). In the wild-type, \(Ptc\) was posteriorly expressed in an area surrounding the ZPA (Fig. 3G). In \(Gli3^{−/−}\) limb buds, \(Ptc\) was also expressed in an anterior domain, which corresponded to the ectopic anterior \(Shh\) domain (Fig. 3H). In \(Gli3^{Δ699}\) limbs buds, expression of \(Ptc\) was undetectable in the distal mesenchyme (Fig. 3I). Remarkably, this area of reduced \(Ptc\) expression is congruent with the posterior part of the \(Gli3^{Δ699}\) expression domain (compare Fig. 3I and C).
expression extended anteriorly in the distal mesenchyme (Fig. 4H). In contrast, Gli3\(^{699/699}\) limb buds showed an increased posterior restriction of Hoxd12 expression (Fig. 4I). Next, we looked for the expression of dHand, which is expressed in posterior and distal limb bud mesenchyme at E11.5 (Fig. 4J). dHand expression is upregulated under the influence of SHH signaling (28,29) and is repressed in the anterior limb bud by GLI3 (16). In Gli3\(^{−/−}\) limbs, dHand expression was expanded anteriorly (Fig. 4K). In Gli3\(^{D699/D699}\) limbs, distal expression of dHand was strongly reduced and posterior expression was more restricted (Fig. 4L). Thus, the analysis of AP markers clearly suggested more anterior and less posterior properties of Gli3\(^{D699/D699}\) limbs and thus confirmed the interpretation of morphological criteria.

Analysis of molecular digit identity

Hoxd12–13 are expressed in the forming autopod of mice and are thought to impart a dose-dependent mechanism for the proliferation and growth of phalangeal structures (30,31). At E12.5, the region of digit 1 identity in the murine autopod is characterized by Hoxd13 expression and the absence of Hoxd12 expression (10). At E12.5, embryos of all three genotypes showed Hoxd13 expression in all digit condensations (Fig. 5A–C). Hoxd12 expression in the wild-type delineated all posterior condensations but was completely absent in the region encompassing the presumptive digit 1 (Fig. 5D). In Gli3\(^{−/−}\) limb buds, Hoxd12 was expressed equally strongly in the region of anterior and posterior digits (Fig. 5E). In contrast, Gli3\(^{D699/D699}\) limbs showed continuous Hoxd12 expression only in the three posterior digit condensations (Fig. 5F).

Apart from more pre-axial digits of anterior-most character, Gli3\(^{D699/D699}\) mutants also did develop malformed posterior digits. Tbx2 and Tbx3 have recently been suggested to contribute to the formation and specification of posterior-most digits in vertebrate limb development (32–34). In the wild-type, Tbx2 expression is strong in the region of prospective digit 5 and weak in region of prospective digit 4 (Fig. 5G). Whereas autopodial Tbx2 expression in Gli3\(^{−/−}\) limbs was surprisingly similar to wild-type (Fig. 5H), expression in Gli3\(^{D699/D699}\) was found to be completely absent in the region of presumptive digit 4 and reduced in the region of digit 5 (Fig. 5I). Tbx3 is expressed in an anterior and a posterior domain in wild-type limbs at E11.5 (Fig. 5J). Lack of GLI3 in Gli3\(^{−/−}\) limbs led to an exclusive loss of anterior Tbx3 expression, whereas the posterior expression persisted (Fig. 5K). In contrast, anterior Tbx3 expression was normal in Gli3\(^{D699/D699}\) limbs, whereas the posterior distal domain was clearly reduced (Fig. 5L). Hence, the molecular analysis of Gli3\(^{D699/D699}\) limb buds revealed intact anterior gene expression and increased repression of genes, which are
proposed to contribute to the specification of posterior digit identities.

DISCUSSION

PHS is a pleiotropic disorder of human development that comprises a multitude of symptoms ranging from skeletal displasia to life-threatening malformations of the inner organs (for a summary of PHS symptoms, please refer to http://www.nlm.nih.gov/archive/20061212/mesh/jablonski/cgi/jablonski/syndrome_cgifa17.html). The majority of symptoms is extensively phenocopied by the GLI3-D699 mutant (13). However, whereas PHS is inherited as a dominant trait, symptoms in the mouse are observed only in homozygous embryos. One obvious explanation is a species difference in dosage sensitivity resulting in different penetrance of the phenotypes, as has been reported for several other genes, such as GATA3, LMX1b, MSX2 and TBX1. In all these cases, heterozygous effects are evident in humans but not in mice, and the homozygous effects in mice are similar in nature to the human heterozygous effects (35–38). Equally, the abnormalities of PHS patients and homozygous GLI3-D699 mutant mice are of the same type and affect mostly the same organs in both cases.

An especially striking degree of congruence is to be found in the development of the limbs. Both human and murine disease patterns comprise short limbs, polydactyly, oligodactyly and dysplastic digits. In accordance with clinical reports on human patients, oligodactyly was more rarely observed than polydactyly in mutant mice. Thus, oligodactyly seems to constitute the more severe outcome of limb development that is under the influence of either GLI3PHS or GLI3-D699. In a normal limb, digit length peaks at the central position of the autopod. Consequently, the silhouette of the oligodactylous fore limbs of GLI3-D699/D699 embryos strongly suggested that the absence of the posterior-most digits was the cause for oligodactyly. This is partially reminiscent of the ulnar-mammary syndrome in human patients who have mutations in the TBX3 gene (32). Among other symptoms, this congenital disorder is characterized by the absence or deformation of digit 4 and/or digit 5. Chick studies have further supported the importance of both TBX2 and TBX3 for the specification and formation of the posterior digits (34). Expression of dominant negative forms of TBX2 and TBX3 caused anterior transformation or even the complete loss of the two posterior-most digits (34). These results reveal close similarity to what we have seen in GLI3-D699/D699 embryos. Thus, on the one hand, the increased repression of posterior Tbx2 and Tbx3 expressions in GLI3-D699/D699 embryos provides a likely explanation as to why the posterior digits of polydactylous limbs are considerably malformed. Secondly, given the persistence of the pre-axial duplications of anterior digit identities even in the oligodactylous limbs, it is plausible that oligodactyly is merely the most severe result of the anteriorizing effect of GLI3-D699, which, in these cases, results in the most drastic malformation of posterior digits, namely their complete absence.

In accordance with the more frequently reported polydactyly of PHS patients, however, the majority of GLI3-D699/D699 embryos phenocopies the polydactylous end of the PHS disease pattern. As yet, PHS associated polydactyly has been very difficult to understand regarding the cause of the disease. PHS originates from mutations, which are believed to yield a truncated GLI3 protein that might act as a constitutively active GLI3R (2,7,12,39). Since it has been shown that the removal of GLI3 is capable of rescuing the nearly digitless phenotype of Shh-/- mice, it is believed that GLI3R exerts a potent negative effect on digit formation (8,11). Consequently, PHS causative mutations should theoretically be expected to result in a substantial reduction of digit number.

This controversy could be interpreted in different ways. For instance, it might have posed the question of possible divergent functions of GLI3 during murine and human limb development. However, our initial analysis of GLI3-D699 has already revealed that this analogous mutation in mice phenocopies the majority of PHS associated symptoms, including some characteristic alterations of limb development (13). The therein reported central position of polydactyly was not observed in the course of this study, most likely because of the establishment of a homogenous genetic background as a result of prolonged backcrossing into C57BL/6 mice. Nevertheless, our new data now exposed previously unreported phenotypic overlaps of GLI3-D699/D699 embryos and PHS patients. Recently, radiological examination of severely affected patients showed absent proximal phalanges in the hands and shortened metacarpals and phalanges of the second through fifth digits (40). This clinical description is strongly reminiscent of the phenotype of GLI3-D699/D699 embryos, which develop an increased share of biphalangeal short digits. It appears that inbred mice may reflect only one side of the phenotypic spectrum observed in the heterogenous population in man. In this regard, the GLI3-D699 mutation in the C57BL/6 mouse line may depict the severe cases of PHS in man. Nevertheless, the theory of grossly divergent functions of GLI3 within human and murine limb development can certainly be abandoned.

Instead, our analysis of GLI3-D699 mutants implies a different approach to the reconciliation of PHS associated polydactyly with the current understanding of GLI3R function. We could demonstrate that GLI3-D699 possesses a number of functions, which have been proposed for the endogenous GLI3R protein. GLI3-D699 indeed exerts a certain repressor activity on SHH target genes such as Ptc1. Furthermore, GLI3R is believed to be necessary for digit 1 identity while interfering with the formation of posterior digit identities (10,41). Accordingly, homozygous GLI3-D699 embryos develop additional digit 1 and display malformation and/or absence of posterior digits. We could show that GLI3-D699 indeed represses posterior gene expression as is predicted for GLI3R (8,11,38). Furthermore, presumably through the restriction of posterior gene expression, GLI3-D699 efficiently promotes anterior expression of genes, which have been shown to absolutely require GLI3R function (16,25,37). Obviously, GLI3-D699 executes GLI3R function within the early regulatory network of AP patterning.

Still, our analyses also uncovered an unexpected discrepancy between GLI3-D699 and the general image of GLI3R. High levels of GLI3R are supposed to interfere with the maintenance of the SHH/FGF feedback loop, which is essential for proper outgrowth of the limb bud (8,11,15,20). GLI3-D699, on the other hand, fails to reveal an increased potential in repressing the members of the feedback loop, particularly Gremlin
and Fgf8. On condition that current theories concerning GLI3R function are fully correct, it appears that PHS causative mutations, which here are mimicked by Gli3\textsuperscript{D699}, result in a somehow GLI3R-like but not GLI3R-equivalent protein. In that scenario, GLI3\textsuperscript{PHS}/GLI3\textsuperscript{D699} would exhibit some but not all functions that have so far been proposed for the endogenous GLI3R. That result is not only of importance in broadening our understanding of PHS pathogenesis, but it also reveals the necessity of a more cautious interpretation of previously performed analyses of artificially truncated GLI3 proteins (7,42–44). For example, transfection experiments in cell culture have never been able to show that the function of truncated GLI3 proteins of various sizes may be inequivalent to one another or to the endogenously produced GLI3R. Possibly, the vast overexpression of GLI3 in these experimental settings, considerably exceeding physiological levels, has made it infeasible to appreciate subtle functional differences.

Whether the discrepancy between GLI3\textsuperscript{D699} and GLI3R might be ascribed to additional modifications of GLI3R in the course of the normal processing procedure or whether it is based on other molecular differences between GLI3\textsuperscript{D699} and the molecularly still undefined GLI3R remains to be elucidated. In addition, it is still conceivable that the current perceptions of GLI3R function are not fully comprehensive since they are mostly based on studies of SHH-deficient mouse mutants. For example, even in limb development, it might be that the role of SHH comprises more than just the regulation of GLI3 processing alone. We are planning to tackle these open questions by analyzing GLI3\textsuperscript{D699} in combination with an SHH deficiency and by investigating the in vivo effect of further truncated GLI3 proteins, which are either smaller or larger than GLI3\textsuperscript{D699}.

MATERIALS AND METHODS

Bone and cartilage staining

Embryos were fixed in 80% ethanol, skinned and eviscerated. Subsequently, they were dehydrated in ethanol overnight, degreased in acetone overnight and stained in Alcian Blue/Alizarin Red overnight at 37°C. Embryos were then rinsed with ethanol for 1 h and finally were run through 1% potassium hydroxide:glycerol series and stored in pure glycerol.

Whole-mount in situ hybridization

Whole-mount in situ hybridizations were performed essentially as described previously (45). Probe information can be provided on request.

Mice

Gli3\textsuperscript{D699} and Gli3\textsuperscript{XJ} mice were maintained as heterozygous stocks. Embryonic day 0.5 was the day vaginal plugs were found. Embryos were harvested between E10.5 and E19.5, and genotyping was performed on yolk sac DNA by polymerase chain reaction (PCR). Primer information can be provided on request.

Western blot

Protein lysates were prepared from E10.5 wild-type, Gli3\textsuperscript{D699/+} and Gli3\textsuperscript{D699/D699} whole embryos. Equal amounts of protein were loaded onto 7% SDS–PAGE gels. Immunoblotting was performed as described (7).

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