6 Discussion

6.1 Reduced HS synthesis in *Ext1*$^{Gt/Gt}$ mice

Endochondral ossification is controlled by a complex network of signal interactions (Horton, 1993). To date, little is known about the functional role of the ECM in regulating this process. HSPGs are main structural components of the ECM in cartilage. In addition, they play important roles in regulating signal propagation of various growth factors. To analyze the role of HSPGs in regulating chondrocyte differentiation, we have investigated mice carrying a gene trap insertion in *Ext1*, a glycosyltransferase necessary for the synthesis of HS.

In contrast to *Ext1-/-* mice, which die during gastrulation (Lin et al., 2000), *Ext1*$^{Gt/Gt}$ mice survive until midgestation, revealing a residual function of the mutant *Ext1* allele. Correspondingly, reduced amounts of HS can be detected in *Ext1*$^{Gt/Gt}$ mutant embryos by immunohistochemistry, whereas no HS is synthesized in *Ext1-/-* mice. Similarly, a parallel study revealed that embryonic fibroblasts from *Ext1*$^{Gt/Gt}$ mice produce about 18% HS compared to wild-type cells. The lesser amounts of HS are primarily due to shortened rather than to decreased numbers of HS chains. Interestingly, the sulfation pattern of the mutant HS seems to be normal, suggesting that interactions with growth factors are not perturbed in general (Yamada et al., 2004).

The residual glycosyltransferase activity in *Ext1*$^{Gt/Gt}$ mice could theoretically be contained in the truncated Ext1 fusion protein. It has been shown, however that nonsense and missense mutations scattered throughout the *EXT1* gene lead to similar HME phenotypes in humans (Zak et al., 2002). Furthermore, a murine cell line carrying a splice mutation, which leads to a truncation of the Ext1 protein shortly behind exon1, lacks any HS polymerase activity (McCormick et al., 2000). It is therefore not likely that the truncated *Ext1*$^{Gt/Gt}$ protein, consisting only of the polypeptide encoded by exon1, would maintain sufficient glycosyltransferase activity for the synthesis of HS in *Ext1*$^{Gt/Gt}$ mice. On the other hand, functional Ext1 protein could be generated by alternative splicing around the gene trap vector. By quantitative RT-PCR, we detected about 3% full-length *Ext1* transcripts in homozygous *Ext1*$^{Gt/Gt}$ mice. Low amounts of wild-type Ext1 protein might thus produce sufficient amounts of HS to allow survival until E16.5. Different levels of

alternative splicing in mutant mice may then contribute to the variability of the *Ext1*$^{Gt/Gt}$ phenotype.

6.2 *Ext1*-dependent HS regulates Ihh signaling

The expanded zone of proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mice, which reflects a delay in the onset of hypertrophic differentiation, resembles that of mice overexpressing Ihh under the *Col2a1* promoter. In contrast to *Col2a1-Ihh* mice, we found reduced expression of *Ihh* in *Ext1*$^{Gt/Gt}$ mice. Nevertheless, *Pthlh* expression is upregulated, indicating either Ihh-independent regulation of *Pthlh* expression or increased Ihh signaling. To differentiate between these possibilities, we have investigated Ihh signaling at different levels: First, analysis of *Ptch* expression, a direct target of Ihh signaling (Goodrich et al., 1996), revealed an extended domain of strong *Ptch* expression in proliferating chondrocytes in *Ext1*$^{Gt/Gt}$ mice. Second, by immunohistochemistry we detected a broader domain of Ihh protein in the proliferating chondrocytes of mutant mice. Third, chondrocyte proliferation is upregulated in periarticular chondrocytes similar to mice overexpressing either *Ihh* or an activated form of the Hedgehog receptor *Smoothened* (*Smo*) (Long et al., 2001) under the *Col2a1*-promotor. Fourth, inhibition of Ihh signaling with cyclopamine in *Ext1*$^{Gt/Gt}$ limb explants results in a rescue of the *Ext1*$^{Gt/Gt}$ phenotype. We thus conclude that the range of Ihh signaling is extended toward the distal regions of the cartilage anlagen. As *Pthlh* expression is lost after cyclopamine treatment, we can furthermore exclude an Ihh-independent regulation of *Pthlh* expression in *Ext1*$^{Gt/Gt}$ mice. In summary, our results strongly indicate that upregulation of *Pthlh* expression and the resulting delay in hypertrophic differentiation is due to increased Ihh signaling (Fig. 33). In accordance with these data, a slightly increased distribution of Ihh protein was found in heterozygous *Ext1*+/- mice, which express about 50% HS (M.J. Hilton et al., unpublished data). In contrast, we could not detect a phenotype in mice heterozygous for the hypomorphic *Ext1*$^{Gt/+}$ allele. Together, these data suggest that the distribution of Ihh in the growth plate is dependent on the concentration of HS.

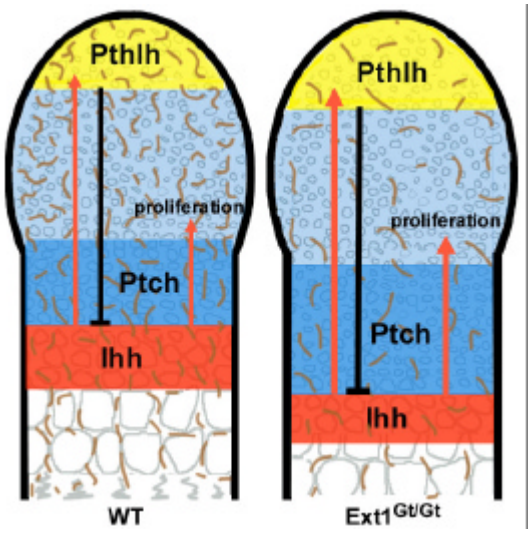

Fig. 33. *Ext1*-dependent HS regulates Ihh signaling. *Ihh*, expressed in prehypertrophic chondrocytes (red), travels through the proliferating chondrocytes to directly activate the expression of *Pthlh* (yellow). Ihh signaling induces strong *Ptch* expression (dark blue) in columnar chondrocytes flanking the *Ihh* expression domain and weaker *Ptch* expression in distal chondrocytes including the periarticular, *Pthlh*-expressing cells (light blue and yellow). HS (brown) negatively regulates the propagation of the Ihh signal. Reduced levels of HS in *Ext1*$^{Gt/Gt}$ mutants facilitate Ihh transport and lead to an increased domain of strong *Ptch* expression and an upregulation of *Pthlh* expression. Pthlh in turn delays the onset of hypertrophic differentiation.

A functional link between HS and Hedgehog signaling was first implicated by analysis of the *Drosophila* mutant *ttv* (Bellaiche et al., 1998; The et al., 1999). Recently, two other members of the *Ext* family have been identified in *Drosophila*: *Sister of ttv* (*sotv*), a homolog of *Ext2*, and *brother of ttv* (*botv*), the fly *Ext-like 3* homolog, a more distantly related member of the Ext family (Takei et al., 2004). Mutations in either gene lead to loss or severely reduced amounts of HS. Consequently, in clones of mutant cells in the imaginal discs, hh target genes are activated in one row of cells directly flanking the *hh* expression domain but not in cells located several cell diameters away from the source of hh expression (Han et al., 2004; The et al., 1999). These results indicate a role for ttv-dependent HS in transporting hh in the extracellular space.

Based on the *Drosophila* studies, it might be expected that the reduced levels of HS in *Ext1*$^{Gt/Gt}$ mice would result in decreased Ihh signaling and, hence, loss of *Pthlh* expression and accelerated hypertrophic differentiation. In contrast, our results clearly

demonstrate that Ihh signaling is increased, not decreased. As *Ext1*$^{Gt/Gt}$ mice produce lower amounts of HS, we conclude that HS restricts Ihh propagation in mice, thereby negatively regulating Ihh signaling. This hypothesis is supported by treatment of wild-type limb explants with HS, which leads to a concentration-dependent restriction of *Ptch* expression to cells flanking the *Ihh* expression domain. Interestingly, treatment with heparin, a highly sulfated subclass of HS, shows an even stronger restriction of the Ihh signal indicating that not only the amount of HS but also the degree of sulfation might be critical to determine the range of the Ihh signal.

Treatment of limbs, which overexpress Ihh in chondrocytes, revealed that high levels of HS do not influence expression of *Pthlh* or the onset of hypertrophic differentiation in these transgenic mice. Thus the negative regulation of Ihh signaling by HS seems to be due to a restriction of Ihh protein distribution instead of inhibiting binding of the Ptch receptor. As CS has no effect on the domain of *Ptch* expression, the restriction in Ihh signaling activity by HS reflects a specific regulatory process.

The role of HS in regulating Ihh signaling in mice seems to be in contrast to its proposed function in regulating hh in *Drosophila*. This discrepancy could implicate a different role of HS in regulating Hedgehog signaling in vertebrates and flies. Given the conservation of the Hedgehog signaling pathway, however, it is more likely that the difference reflects the different alleles investigated, null in *Drosophila* and hypomorphic in mice. We thus propose a dual function for HS in controlling Hedgehog signals: First, HS is necessary to bind Hedgehog molecules in the extra cellular space, thereby facilitating transport of the Hedgehog signal from cell to cell. In addition binding of Hedgehog molecules to HS might prevent their degradation. Total loss of HS as in *ttv* mutants or *Ext1*-/- mice would then result in loss of biological available Hedgehog protein and consequently in a loss of Hedgehog signaling. Second, increasing concentrations of HS sequester increasing amounts of Hedgehog molecules, thereby restricting Hedgehog activity in a concentration-dependent manner to the source of its expression domain. Fine-tuning the levels of HS would thus provide an important mechanism to regulate the range of Hedgehog acting as a morphogen. In this context it would be interesting to study a conditional loss of function mutation for *Ext1* in chondrocytes of the growth plate, to

analyse if Hedgehog transport is completely abrogated and to analyse hypomorphic alleles in *Drosophila*.

A similar function for HSPGs in restricting growth factor signaling has been proposed for the regulation of dpp and wg signaling in *Drosophila*. Overexpression of *dally* or *dally-like*, the two *glypican* homologs in *Drosophila*, restricts wg and dpp proteins to the site of their expression (Baeg et al., 2001; Fujise et al., 2003). These data suggest that an excess of HSPGs negatively regulates the distribution of different growth factors.

Although it is widely accepted that Hedgehog molecules can form activity gradients in the wing imaginal disc in *Drosophila* and in the neural tube in vertebrates, it is not clear how this posttranslationally modified molecule moves through the tissue. Several models have been put forward to explain these processes and most of these do not exclude a role for HS in transporting this lipid modified molecule. The simpliest model, the diffusion model, predicts that morphogen molecules secreted from their source diffuse through the extracellular space, creating a gradient of concentration. However the fact that the Hedgehog molecule is lipid modified and might therefore be membrane associated, is hardly reconcilable with the simple diffusion model. In addition our analysis of the role of HS in the developing growth plate revealed that GAGs influence the distribution of Ihh in a concentration dependent manner. HSs bind to Hedgehog molecules directly, determining the concentration of this molecule in a certain area. Similarly, during mouse cerebellum development it was shown that high levels of HS are present at specific developmental time points in the external granule cell layer, which correlate with an accumulation of Shh in this tissue at the same time (Rubin et al 2002). The ‘argosome’ model suggest that membrane pieces vesiculate, containing the lipid modified molecules, which than travel through the tissue (Greco et al., 2001). In a similar model the formation of hedgehog particles, ‘Large Punctate Structures’ (LPS), were postulated (Gallet et al., 2003). Possibly these hedgehog vesicles or LPS are transported by binding to a HSPG, which then passes the signaling molecules to another HSPG on an adjacent cell (Nybakken and Perrimon, 2002). More analysis in *Drosophila* and vertebrates will be needed to clarify the transport mechanism of this interesting molecule.

In addition to regulating protein distribution, HS mediates binding of Fgfs to their receptors (Esko and Selleck, 2002). It has still to be resolved if HS is required for binding

of Ihh to its receptor. In *Drososphila*, clones mutant for *dally* and *dally like*, *ttv* or *sulfateless*, a protein necessary for the sulfation of HS, can receive Hedgehog signals and activate Hedgehog target genes in one row of cells flanking the source of hh. However they cannot transport the signal into the neighboring tissue (Bellaiche et al., 1998; Han et al., 2004). Therefore receptor binding seems to be HS independent. Further studies are needed to determine whether binding of hh to its receptor in these clones is dependent on HS from neighboring wild-type cells. In our limb explant studies, addition of HS fails to affect the upregulation of Ihh target genes in limbs overexpressing *Ihh*. These data support a role of HS in regulating the distribution rather than the reception of Ihh signals.

6.3 Direct regulation of *Pthlh* by Ihh

As previously explained, it has not been resolved if Ihh signals directly act on the Pthlh promoter or if secondary signals like Bmps or Tgfs are needed (Alvarez et al., 2002; Zou et al., 1997). Recent experiments have excluded Bmps from mediating the Ihh signal (Minina et al., 2001). Similarly, we show here that Tgfb1 cannot induce *Pthlh* expression in an Ihh-independent way in midgestation embryos, a result that differs from previous studies (Alvarez et al., 2002) and might reflect stage-specific differences. Instead, the data presented in this study, in combination with experiments from other laboratories, strongly implicate a direct role of Ihh in regulating *Pthlh* expression (Fig. 33). First, we and others (Gritli-Linde et al., 2001) have shown by immunohistochemistry that Ihh can travel over long distances in the developing cartilage anlagen. The region, in which we could detect Ihh protein roughly correlates with the domain of strong *Ptch* expression, reflecting strong Ihh signaling activity. Second, we detected weak but significant expression of *Ptch* at the distal ends of the skeletal elements including those chondrocytes that express *Pthlh*. In addition, in some $Ext1^{Gt/Gt}$ embryos, *Ptch* expression is strongly upregulated in cells expressing *Pthlh* (Fig. 12), supporting a direct regulation. Third, treatment of limb explants with ectopic HS restricts *Ptch* expression to cells directly flanking the *Ihh* expression domain. These cells are the most likely source to express a secondary signal. However, no *Pthlh* expression can be detected in periarticular cells. Although we cannot completely exclude that HS restricts the distribution of a hypothetical secondary signal similar to that of Ihh, our results are most parsimonious with Ihh acting as a long-range signal that directly activates *Pthlh* expression. Fourth, the function of *Smo* has recently been disrupted in

Col2a1-expressing chondrocytes (*Col2a1-SmoC*) (Long et al., 2001). Unexpectedly, chondrocyte differentiation is not affected in these mutants. In wild-type embryos, *Pthlh* is expressed in *Col2a1*-expressing periarticular chondrocytes (Fig. 16). In contrast, in *Col2a1-SmoC* mice, cells in the joint region, which do not express *Col2a1*, highly express *Pthlh*, whereas no expression can be detected in the most distal chondrocytes (Figure 5 in Long et al., 2001). The shift of *Pthlh* expression to cells outside the *Col2a1* expression domain in *Col2a1-SmoC* mutants strongly supports a direct activation by Ihh. These results implicate that Ihh can travel over long distances in cartilage to form a morphogen gradient. Different levels of Ihh would then activate different sets of target genes, thereby controlling specific steps of chondrocyte differentiation.

6.4 HS and Fgf Signaling

Our results indicate that potentiation of Ihh signaling might be the main cause for the $Ext1^{Gt/Gt}$ phenotype. It is, however, possible that other signaling systems are also affected. As HS stabilizes the Fgf ligand-receptor complex (Esko and Selleck, 2002), reduced Fgf signaling might contribute to the delay in hypertrophic differentiation in $Ext1^{Gt/Gt}$ mice. However, the response of $Ext1^{Gt/Gt}$ limbs treated with Fgf in culture indicates that reduced amounts of HS in these mutants do not significantly alter binding of Fgfs to their receptors. In addition, ligand-independent activation of Fgf signaling in compound $Ext1^{Gt/Gt}$;$Fgfr3^{Ach/+}$ mutants cannot rescue the $Ext1^{Gt/Gt}$ phenotype. It is thus unlikely that loss of Fgf signaling is a major cause for the delayed differentiation at the investigated stages. We did, however, observe a reduced degree of elbow fusions in $Ext1^{Gt/Gt}$;$Fgfr3^{Ach/+}$ mutants compared to $Ext1^{Gt/Gt}$ mice. Fgf-dependent inhibition of chondrocyte proliferation might thus prevent secondary joint fusions. Together, these results correlate well with earlier studies placing Fgf signaling upstream to that of Ihh in regulating the onset of hypertrophic differentiation and in parallel in regulating chondrocyte proliferation (Minina et al., 2002). While our analysis of endochondral ossification in $Ext1^{Gt/Gt}$ mutants points to a role for Ext1 in regulating Ihh signaling, we cannot exclude a possible misregulation of additional signaling pathways in other tissues or during other stages of development. In contrast to previous reports, it has recently been shown that mutations in ttv, sotv, and botv affect wg and dpp signals in addition to hh (Takei et al., 2004). Similarly, tissue-specific deletion of *Ext1* using the *nestin* promoter leads to distinct neuronal defects reminiscent of disrupted Fgf,

Wnt, and Slit signaling (Inatani et al., 2003). Signaling through these molecules might thus be affected at later stages or in different organs in *Ext1*$^{Gt/Gt}$ mice. Due to its size, the developing growth plate might be very sensitive to detect slight differences in the propagation of morphogens over long distances, as we could show for Ihh. Analysis of growth factor signaling in cartilage can thus reveal regulatory mechanisms that are masked in smaller tissues.

6.5 Ihh Signaling and the Development of Exostoses

Although mutations in *EXT* genes have been linked to HME, it is poorly understood how they lead to the formation of exostoses. *Ext1* has been classified as a tumor suppressor gene and somatic Loss of Heterozygosity (LOH) or secondary mutations in an *EXT* homolog have been hypothesized to give rise to the isolated exostoses. However, mutation analysis in exostoses tissues of 16 HME patients have detected only one case of LOH, giving limited support for the second hit model (Hall et al., 2002). The reduced levels of HS in *Ext1*$^{Gt/Gt}$ and *Ext1*+/- mice (Lin et al., 2000) suggest that HME patients produce reduced levels of HS, making haploinsufficiency of *Ext1* or *Ext2* the most likely cause for the development of exostoses.

Our results suggest that reduced amounts of HS potentiate Ihh signaling, resulting in delayed hypertrophic differentiation and increased chondrocyte proliferation (Fig. 33). In mice, it has recently been shown that activated signaling through the receptor of Pthlh, Pthr1, which acts downstream of Ihh, results in local enchondroma-like lesions. This skeletal disease is characterized by formation of common benign cartilage tumors, caused by chondrocyte overproliferation and delayed differentiation (Ollier and Maffucci diseases) (Hopyan et al., 2002). Activated Ihh signaling in HME patients might similarly result in clusters of chondrocytes that overproliferate. Groups of proliferating cells in close contact to the perichondrium might then be able to escape the overall regulation of growth plate differentiation by breaking through the perichondrium, thereby inducing the development of an exostosis. Disturbed chondrocyte orientation as observed in *Ext1*$^{Gt/Gt}$ mice might facilitate such a mechanism.

6.6 *Gli* genes have overlapping and distinct expression patterns in the growth plate

Analysing the role of HS in regulating the propagation of the Ihh signal has revealed strong evidence that Ihh acts as a long range signal in the developing cartilage anlagen directly inducing the expression of *Pthlh*. Besides its role in regulating the onset of hypertrophic differentiation, Ihh regulates chondrocyte proliferation and the ossification of the perichondrium in an *Pthlh* independent way. Presumably high levels of Ihh induce chondrocyte proliferation and bone collar formation, whereas low levels of Ihh are sufficient to induce expression of *Pthlh* in periarticular chondrocytes. To further understand how these different functions of Ihh are translated, we analysed the expression patterns of the three downstream transcription factors *Gli1*, *Gli2* and *Gli3* in the growth plate of developing limbs.

All three genes are expressed in overlapping but distinct regions in the growth plate. Together, *Gli2* and *Gli3* are highly expressed in those cells, which are more distant from the Hh source, whereas *Gli1* is highest expressed adjacent to the *Ihh* expression domain. Thus unlike in the neural tube, Gli2 might oppose Ihh signaling during bone development and could possess both: an activator and a repressor function. The best way to elucidate this question would be to use antibodies against the N-terminus or C-terminus of Gli2. Several antibodies were raised against the different epitopes, but unfortunately none of them detected endogenous protein from limb buds (Wang et al., 2000).

Analysis of the expression of all three *Gli* genes in *Ihh* deficient mice, revealed that *Gli2* and *Gli3* transcription does not primarily depend on Ihh. Both genes are expressed in chondrocytes and *Gli3* is additionally expressed in the joint region. The level of *Gli3* expression is unchanged, suggesting that Ihh does not repress *Gli3* transcription in chondrocytes, as it has been described for Shh in the limb bud (Marigo et al., 1996). However *Gli1* is not expressed in *Ihh* deficient limbs indicating that similar as in the neural tube *Gli1* transcription depends on either Gli2- or Gli3-activator function.

6.7 Gli3 acts as a repressor downstream of Ihh signaling

The interaction of Gli3 and Shh has been intensively studied in the developing limb bud and in the neural tube. Shh mutants are characterized by cyclopia, absence of most

ventral cell types along the neuraxis and lack of elements in the autopod and zygopod (Chiang et al., 1996). Remarkably in *Shh;Gli3* and *Smo;Gli3* double mutants, many ventral cell types are rescued and their limbs are distally complete and polydactylous (Litingtung et al., 2002; Wijgerde et al., 2002). Therefore, Gli3 seems to act primarily as a repressor downstream of Shh.

To analyse if Gli3 exerts repressor function downstream of Ihh during endochondral ossification, we compared *Ihh-/-* mice to *Ihh-/-;Gli3-/-* double mutant mice. Our analysis of *Ihh-/-;Gli3-/-* mutants revealed a partial rescue of *Ihh* deficient skeletal elements in a remarkable way: the size of the skeletal elements is increased and distinct zones of periarticular, columnar and hypertrophic chondrocytes are established. Compared to *Ihh-/-* mice two aspects of chondrocyte differentiation are rescued in the double mutants: First *Pthlh* expression is restored in the joint region, leading to a delayed onset of hypertrophic differentiation. *Pthlh* thus seems to be a direct transcriptional target of the Gli3-repressor (Fig. 34). Second, the zone of columnar proliferating chondrocytes is reestablished and cells in this region expresses *Ptch* at low levels. Surprisingly this zone of columnar chondrocytes is of similar size as in wild-type cartilage elements, whereas the zone of periarticular cells is reduced similar to that in *Gli3-/-* mice. Therefore, the differentiation of periarticular chondrocytes seems to be a new differentiation step depending on the action of Gli3-repressor.

Chondrocyte proliferation is regulated by Ihh signaling indepently of Pthlh (Karp et al., 2000). The size of the zone of columnar chondrocytes in *Ihh-/-;Gli3-/-* mice implicates that the proliferation rate is restored. Therefore Gli3 seems to negatively regulate chondrocyte proliferation. The rate of chondrocyte proliferation in *Gli3-/-* mice remains to be analysed. However, the rate does not seem to be upregulated, as the zone of columnar chondrocytes is of similar size as in wild-type skeletal elements. It is therefore likely that chondrocyte proliferation depends on balanced signaling of both: Gli2- and Gli3-activators and Gli2- and Gli3-repressors. Intensive studies in different mouse models will be needed to explore, which combination of factors is responsible for proper regulation of the proliferation rate.

In summary our analysis of *Ihh-/-;Gli3-/-* double mutants suggest that Gli3 acts mainly as a repressor during the process of endochondral ossification. In this study we

could not address, if Gli3 has also an activator function during chondrocyte differentiation. The mild phenotype of *Gli2* deficient mice, however, suggest that Gli3-activator can act downstream of Ihh to positively regulate Ihh target genes. It is not clear either, if Gli2 acts mainly as an activator, like in the neural tube, or if Gli2 has also repressor function during endochondral ossification. Intercrossing of *Gli2-/-* and *Ihh-/-*mice, will be an important experiment, to reveal if Gli2 has any repressor function. If so, loss of *Gli2* in *Ihh-/-* mice should lead to a partial rescue of the *Ihh-/-* phenotype.

6.8 Chondrocyte differentiation is affected in *Gli3* deficient mice

Gli3 deficient mice display a severe patterning defect of the anterior-posteror axis during early limb bud formation. In contrast, chondrocyte and osteoblast differentiation at later stages are only mildly affected in *Gli3* deficient mice. The mutant limbs seem to be slightly delayed in their differentiation process, as their skeletal elements are shorter. Furthermore the zone of proliferating chondrocytes is slightly reduced, which points to an accelerated onset of hypertrophic differentiation. However an accelerated onset of hypertrophic differentiation would imply reduced levels of *Pthlh* or inhibited function of the Pthlh receptor *Pthr1*. Our analysis, however, revealed that expression of *Pthlh* is slightly upregulated instead of downregulated. In addition transcription levels of *Pthr1* are normal in *Gli3-/-* mice (Fig. 28). Therefore shortening of the zone of proliferating chondrocytes might result from a different mechanism. Careful analysis of *Pthlh* expression showed that the expression domain is shifted towards the end of the skeletal elements. Measurement of the distance between the shifted *Pthlh* expression domain and the *Ihh* expression domain in *Gli3-/-* mice revealed, that this zone is of similar size compared to wildtype, indicating, that hypertrophic differentiation is not affected in *Gli3-/-* mice (Fig. 29). Analysis of markers for periarticular chondrocytes (*Fgfr1*) and columnar chondrocytes (*Fgfr3*) revealed that the zone of periarticular chondrocytes (zoneI) is reduced in *Gli3-/-* mice, whereas the zone of columnar chondrocytes (zone II) is of similar size in wild-type and *Gli3-/-* mice. Therefore Gli3 seems to repress the differentiation from periarticular chondrocytes into columnar chondrocytes. Summarizing, the shortened zone of proliferating chondrocytes of Gli3-/- limbs can be ascribed to a reduced zone of periarticular chondrocytes and not to an accelerated onset of hypertrophic differentiation.

To further confirm such a role for the Ihh-Gli3 system, it would be interesting to analyse *Fgfr1* expression in other mouse models, like in *Ihh* overexpressing (*Col2a1-Ihh*) mice.

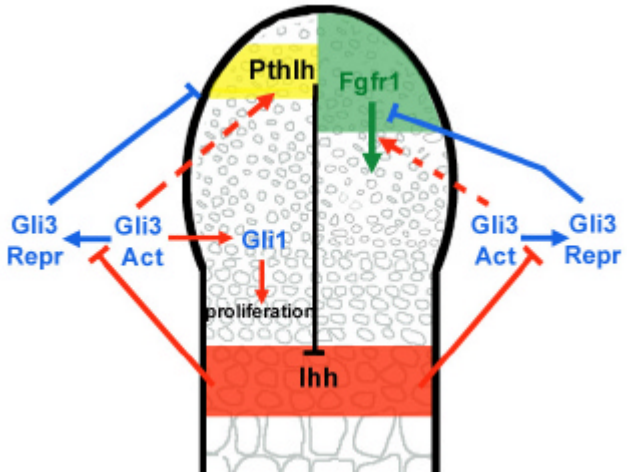

Fig. 34. Preliminary model of the *Gli3* transcription factors acting downstream of Ihh. Ihh inhibits processing of full-lenght Gli3 into the small Gli3-repressor form. In the absence of Ihh signaling, Gli3-repressor downregulates *Pthlh* expression (left side). In addition the Gli3-repressor inhibits the differentiation step from periarticular into columnar chondrocytes (right side, green arrow). In those cells, which receive high levels of Ihh signaling, Gli3 may also act as an activator, inducing the expression of *Gli1* and other Ihh target genes (dashed lines indicate assumed relationsships).

Until today the differentiation from round, low proliferating, periarticular chondrocytes into flat, high proliferating, columnar chondrocyte is not well understood. In this study we have used a new marker, *Fgfr1*, which specifically labels periarticular chondrocytes (zoneI) (Fig. 29). In mice with chondrocyte specific *Pthr1* ablation, chondrocyte differentiation is accelerated not only at the switch between columnar to prehypertrophic chondrocytes but also at an earlier step: the switch from periarticular to columnar chondrocytes (Kobayashi et al., 2002). In these mouse models, Ihh signaling is upregulated in the periarticular region, indicating that Ihh signaling positively control the differentiation of periarticular cells independently of Pthlh (Kobayashi et al., 2002). *Gli3* is highly expressed in periarticular cells. As normally only low levels of Ihh reach this region, Gli3 is likely to be present mainly as repressor form in these cells. Thus Gli3-repressor might inhibit the transition from periarticular into columnar chondrocytes. In *Gli3-/-* mice, Gli3-repressor activity is lost and therefore periarticular cells prematurely develop into columnar cells.