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Trps1, a regulator of chondrocyte proliferation and differentiation, interacts with the activator form of Gli3

Manuela Wuelling [a,1], Frank J. Kaiser [b,1], Laetitia A. Buelens [a], Diana Braunholz [b], Ramesh A. Shivdasani [d,e], Reinhard Depping [c], Andrea Vortkamp [a,*]

[a] Center for Medical Biotechnology, Department of Developmental Biology, University Duisburg-Essen, 45117 Essen, Germany
[b] Institute for Human Genetics, University of Luebeck, 23538 Luebeck, Germany
[c] Department of Physiology, Center for Structural and Cell Biology in Medicine, University of Luebeck, 23538 Luebeck, Germany
[d] Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA 02115, USA
[e] Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, MA 02115, USA

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ABSTRACT

Trps1, the gene mutated in human Tricho-Rhino-Phalangeal syndrome, represents an atypical member of the GATA-family of transcription factors. Here we show that Trps1 interacts with Indian hedgehog (Ihh)/Gli3 signaling and regulates chondrocyte differentiation and proliferation. We demonstrate that Trps1 specifically binds to the transactivation domain of Gli3 *in vitro* and *in vivo*, whereas the repressor form of Gli3 does not interact with Trps1. A domain of 185aa within Trps1, containing three predicted zinc fingers, is sufficient for interaction with Gli3. Using different mouse models we find that in distal chondrocytes Trps1 and the repressor activity of Gli3 are required to expand distal cells and locate the expression domain of Parathyroid hormone related peptide. In columnar proliferating chondrocytes Trps1 and Ihh/Gli3 have an activating function. The differentiation of columnar and hypertrophic chondrocytes is supported by Trps1 independent of Gli3. Trps1 seems thus to organize chondrocyte differentiation interacting with different subsets of co-factors in distinct cell types.

Introduction

Endochondral ossification is a multistep process that starts with mesenchymal cells differentiating into two cell types: chondrocytes, which build the cartilage anlagen, and perichondrial cells surrounding the cartilage model. In the cartilage anlagen two types of proliferating chondrocytes can be distinguished: round, low-proliferating chondrocytes at the distal ends (distal chondrocytes) and high-proliferating chondrocytes that are organized in columns (columnar chondrocytes) towards the center. Columnar chondrocytes that exit the cell cycle differentiate into hypertrophic chondrocytes, which increase in volume and produce a mineralized extracellular matrix. Subsequently, terminal hypertrophic cells are replaced by bone and bone marrow.

One key regulator of endochondral ossification is the secreted signaling factor Indian hedgehog (Ihh), a member of the conserved family of Hedgehog (Hh) proteins. Ihh, which is expressed in prehypertrophic chondrocytes, regulates the onset of hypertrophic differentiation by inducing the expression of *Parathyroid hormone related peptide* (*PTHrP*) in distal chondrocytes (Koziel et al., 2004; Lanske et al., 1996; Vortkamp et al., 1996). PTHrP signals back to the proliferating chondrocytes to inhibit the onset of hypertrophic differentiation and thus, the differentiation of the *Ihh* expressing cell type (Lanske et al., 1996; Vortkamp et al., 1996). In addition, Ihh regulates the rate of chondrocyte proliferation, the size of the zone of distal chondrocytes and the ossification of the perichondrium in PTHrP-independent mechanisms (Karp et al., 2000; Kobayashi et al., 2005; Long et al., 2001; St-Jacques et al., 1999).

In target cells, Hh signals are received by the transmembrane receptor Patched (Ptch1). Binding of Hh to Ptch1 releases the repression of a second transmembrane receptor, Smoothened (Smo), which by a complex signal transduction cascade leads to activation of transcription factors of the Gli-family. In mammals, this family consists of three members, Gli1, Gli2 and Gli3. Gli1 is thought to act mainly as an activator of Hh target genes, while Gli2 and Gli3 can function as activators and repressors. In the presence of Hh signals, Gli2 and Gli3 are thought to enter the nucleus to activate target genes, whereas in absence of Hh signals, Gli3 and, to a lesser extend, Gli2 are proteolytically processed into a short, N-terminal repressor form that inhibits the expression of Hh target genes (Mo et al., 1997; Ahn and Joyner, 2004; Lei et al., 2004; Pan and Wang, 2007). Corresponding to this model, Ihh signaling has been shown to regulate the zone of distal chondrocytes, chondrocyte proliferation and the activation of *PTHrP* expression at least in part by inhibiting

* Corresponding author.
E-mail address: andrea.vortkamp@uni-due.de (A. Vortkamp).
[1] These authors contributed equally to this work.

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the repressor function of the downstream transcription factor Gli3 (Hilton et al., 2005; Koziel et al., 2005; Mau et al., 2007).

The transcription factor TRPS1 has been identified as the gene mutated in patients with Tricho-Rhino-Phalangeal-syndrome (TRPS), an autosomal dominant inherited disorder characterized by developmental defects of facial and skeletal bones (Giedion, 1966; Momeni et al., 2000). Characteristic features of the skeleton of TRPS patients include short stature, hip malformations and cone-shaped epiphyses of phalangeal bones. Based on the clinical phenotype and cytogenetic analyses, TRPS can be classified into three subtypes (Ludecke et al., 2001). Type I represents the mildest form of TRPS and is caused by null mutations or deletions of the *TRPS1* gene. Type III is characterized by brachydactyly and short stature resulting from missense mutation in the GATA-type zinc finger of TRPS1. Type II is a microdeletion syndrome affecting at least *TRPS1* and the neighboring *EXT1* gene. In addition to typical TRPS symptoms Type II patients develop multiple exostoses and other malformations characteristic for mutations in *EXT1* (Ludecke et al., 2001).

TRPS1 encodes a multi-zinc finger protein of 1281 amino acids (aa) with nine predicted zinc finger domains of four different types. The first three zinc fingers share a $C_2X_{14}H_2$consensus sequence, while the zinc finger motifs 4–6 are of the $C_2X_{12}H_2$-type. Until now, no homology or function has been assigned to these domains. The seventh zinc finger motif of TRPS1 represents a DNA-binding, GATA-type zinc finger. This domain binds to GATA consensus sequences and acts as a repressor of GATA-dependent activation of transcription (Malik et al., 2001). The two C-terminal zinc fingers of TRPS1 are of the Ikaros-type. Binding of this domain to cofactors has been show to regulate the transcription activation capacity of Trps1 (Kaiser et al., 2007, 2003a,b).

During mouse embryogenesis *Trps1* expression is first detectable in visceral tissues at E7.5. From E12.5 on *Trps1* is highly expressed in skeletal tissues and hair follicles. In developing endochondral bones *Trps1* is expressed in all proliferating chondrocytes. Strong expression is found in joints, distal chondrocytes and at the border of proliferating and hypertrophic chondrocytes (Kunath et al., 2002). To analyze the function of Trps1, transgenic mice have been generated that harbor an in-frame deletion of exon 4, which encodes the GATA-type zinc finger (Malik et al., 2001). This deletion mimics the consequence of a splice site mutation identified in a TRPS Type I patient (Ludecke et al., 2001, Kaiser et al., 2004). Although mRNA expression and nuclear localization of the mutant protein are preserved, the DNA-binding capacity is deleted in these mutants (Kaiser et al., 2004; Malik et al., 2001). *Trps1* heterozygous mice display facial abnormalities that resemble aspects of the human phenotype. In addition, they develop kyphoscoliosis and structural defects of endochondral bones. Bone histomorphometry revealed reduced bone density and impaired trabecular morphology (Malik et al., 2002). Homozygous mutants are dwarfed and die perinataly due to respiratory problems resulting from malformations of the rib cage (Malik et al., 2002). Recently, reduced chondrocyte proliferation has been described in *Trps1*$^{-/-}$ mutants (Napierala et al., 2008; Suemoto et al., 2007). Furthermore, Trps1 has been shown to interact with Runx2, a main regulator of chondrocyte differentiation, thereby inhibiting Runx2 dependent transcriptional activation (Napierala et al., 2005, 2008).

Here we provide evidence for a direct interaction between Trps1 and Gli3. Interestingly, Trps1 specifically binds to the activator form of Gli3, whereas no interaction of Trps1 with the Gli3 repressor could be detected in overexpression studies or *in vivo*. Trps1 and Gli3 regulate the size of the zone of distal chondrocytes and restrict the zone of *PTHrP* expression in distal cells. Furthermore, analysis of *Trps1*$^{-/-}$*;Gli3*$^{-/-}$ and *Trps1*$^{-/-}$*;Col2-Ihh* mice revealed a cooperative function of Trps1 and Ihh/Gli3 signaling in activating chondrocyte proliferation.

Materials and methods

Transgenic mice

Heterozygous *Trps1*$^{+/-}$ mice (Malik et al., 2002) were maintained on a C57Bl/6J genetic background and crossed to either *Gli3*^{Xtj} (Hui and Joyner, 1993), *Col2-Gal4* or *UAS-Ihh* transgenic mice (Long et al., 2001) to generate compound mutants. All animal studies were undertaken according to institutional guidelines. PCR was performed on genomic tail DNA as described previously (Koziel et al., 2005). For *Trps1*$^{-/-}$ mutants, the following primers were used: *Trps1*WT-F: 5′AGTGTTC-CAGTCTCGAAGCCA3′, *Trps1*WT-R: 5′GGTCTGAATTGCGCAAACACC3′, and *Trps1*Neo-R: 5′ATCAGGATGATCTGGACGAAG AG3′. Wild-type mice (NMRI) were obtained from Charles River Laboratories.

Morphological analysis and in situ hybridization

Safranin-Weigert staining and *in situ* hybridization were carried out as described previously (Minina et al., 2002). References for *in situ* hybridization probes are as follows: *Trps1* (Kunath et al., 2002), *Ihh* (Bitgood and McMahon, 1995), *PTHrP (Pthlh — Mouse Genome Informatics)*, (Koziel et al., 2004), *Ptch1* (Goodrich et al., 1996), *Bmp7*, *Fgfr1* and *Fgfr3* (Minina et al., 2005), *Gli3* (Hui and Joyner, 1993), and *Ucma* (Tagariello et al., 2008).

Proliferation analysis

Mice were sacrificed 2 h after receiving intra-peritoneal injections of 50 μg/g body weight of 5-bromo-2′-deoxyuridine (BrdU). Forelimbs were fixed in 4% paraformaldehyde, embedded in paraffin and sectioned. Proliferating cells were detected with the BrdU labeling and detection kit II (Roche) and an Alexa488 labeled secondary antibody (Molecular Probes) according to the manufacturer's instructions. BrdU positive cells were counted in defined regions in distal cells adjacent to the joint and in columnar chondrocytes flanking the *Ihh* expression domain, which was determined by *in situ* hybridization on a parallel section. Regions were defined to cover the maximal width of the ulna in Trps1 mutants. The same seized region was analyzed in all sections. Total cell number was determined after counterstaining with DAPI.

Imaging and statistical analysis

Fluorescence pictures were taken on a Zeiss Axiovert 200 microscope with a Spot 23.0 camera (Diagnostik Instruments) and Metamorph imaging software (Visitron Imaging Systems). BrdU positive cells were evaluated with the Metamorph analysis software using the same settings and signal intensity threshold for all sections. The data were verified by manually counting BrdU and Dapi positive cells in a double blind approach by two independent researchers. For statistical analysis mean values were determined from 3–6 sections per animal of 5 individual mice with a specific genotype for single mutants or 3 individual mice for double mutants. Statistical significance was assessed by an unpaired, two-tailed Student's *T*-tests, p-values <0.05 were regarded as significant. Standard deviation (sd) for all parameters is given as error bars in each histogram.

Cell culture

Cos7 cells were maintained in DME-medium with 10% fetal calf serum. Human hGLI3 activator and hGLI3 repressor (Ruiz i Altaba, 1999) expression plasmids were transfected with Lipofectamine (Invitrogen) or Genepulsing (BIORAD) following standard procedures provided by the manufacturers. For GST pull-down analysis, two different fragments of the GLI3 transactivator domain coding for aa 745–1580 and aa 827–1580, respectively, were subcloned in a pGEX-

4T-2 vector (Clontech) and expressed as Glutathione S-transferase (GST) fusion protein.

Co-immunoprecipitation and GST pull-down assay

Protein extracts of Cos7 cells were prepared as described previously (Kaiser et al., 2003a). Limbs skeletal elements from E16.5 wild type NMRI, *Trps1*$^{-/-}$ and *Trps1*$^{+/+}$ mice were dissected from surrounding skin and muscles and grinded in liquid nitrogen prior to resuspension in RIPA lysis buffer. Western blot and co-immunoprecipitation were performed with primary antibodies against c-myc, Gli3 (Santa Cruz Biotechnology, Hu et al., 2006), hTRPS1 (Chang et al., 2002; Kaiser et al., 2003a,b), M2-FLAG (Sigma) and horseradish peroxidase conjugated secondary goat anti-rabbit (Dako) or rabbit anti-mouse (Santa Cruz/USB) antibodies. The mouse Trps1 antibody was generated against the same N-terminal 15 amino acids (aa1–aa15) of the mouse protein (Eurogentec) corresponding to those used to generate the human TRPS1 antibody. To verify the specificity of the mouse Trps1 antibody, tissue extracts from wild type mice were tested on western blot with the α-mTrps1 and a-hTRPS1 antibodies. Both antibodies detected the same band of Trps1 at 160 kDA (Fig. 8C and Sup. Fig. 2D). Chemiluminescense detection (Pierce) was performed according to the manufacturer's instructions. GST pull-down assays were performed as described earlier (Depping et al., 2008). Briefly, 15 μg purified GST or GST-fusion proteins were allowed to bind to Glutathione-sepharose 4B (GE Healthcare Bio-Sciences) at 4 °C for 1 h. GST pull-down assays were carried out with purified GST as a negative control. TRPS1 fragments were transcribed and translated *in vitro* (TNT Coupled Reticulocyte Lysate System) in the presence of [35S]-methionine (Hartmann Analytic) according to the manufacturer's protocol. 10 μl of the reaction batch were allowed to bind to the immobilized GST fusion-proteins. Proteins were separated by SDS-PAGE (10–15%). To detect the [35S]-labeled proteins, the dried gels were autoradiographed (15 h).

Results

Reduced size of the skeletal elements in Trps1$^{-/-}$ *mutants*

In order to characterize the skeletal phenotypes observed in *Trps1*$^{-/-}$ mice we first conducted morphological analysis on limb sections of *Trps1*$^{-/-}$ mutants. At E12.5 *Trps1*$^{-/-}$ mutant skeletal elements are indistinguishable from those of wild type littermates (Sup. Figs. 1A, B). At E13.5 hybridization with *Collagen Type X (ColX)* revealed a slightly delay in the onset of hypertrophic differentiation (Sup. Fig. 1F, red arrow). This delay is more obvious at E14.5, when the two *ColX* expression domains are not separated in the mutants (Sup. Fig. 1H). At E16.5 ulna and radius of *Trps1*$^{-/-}$ mutants are significantly shorter (Fig. 1C) and thinner (Fig. 1F) than those of wild type littermates (Figs. 1A, D). Measurements of *Trps1*$^{-/-}$ ulnae revealed a reduction to 80% in length

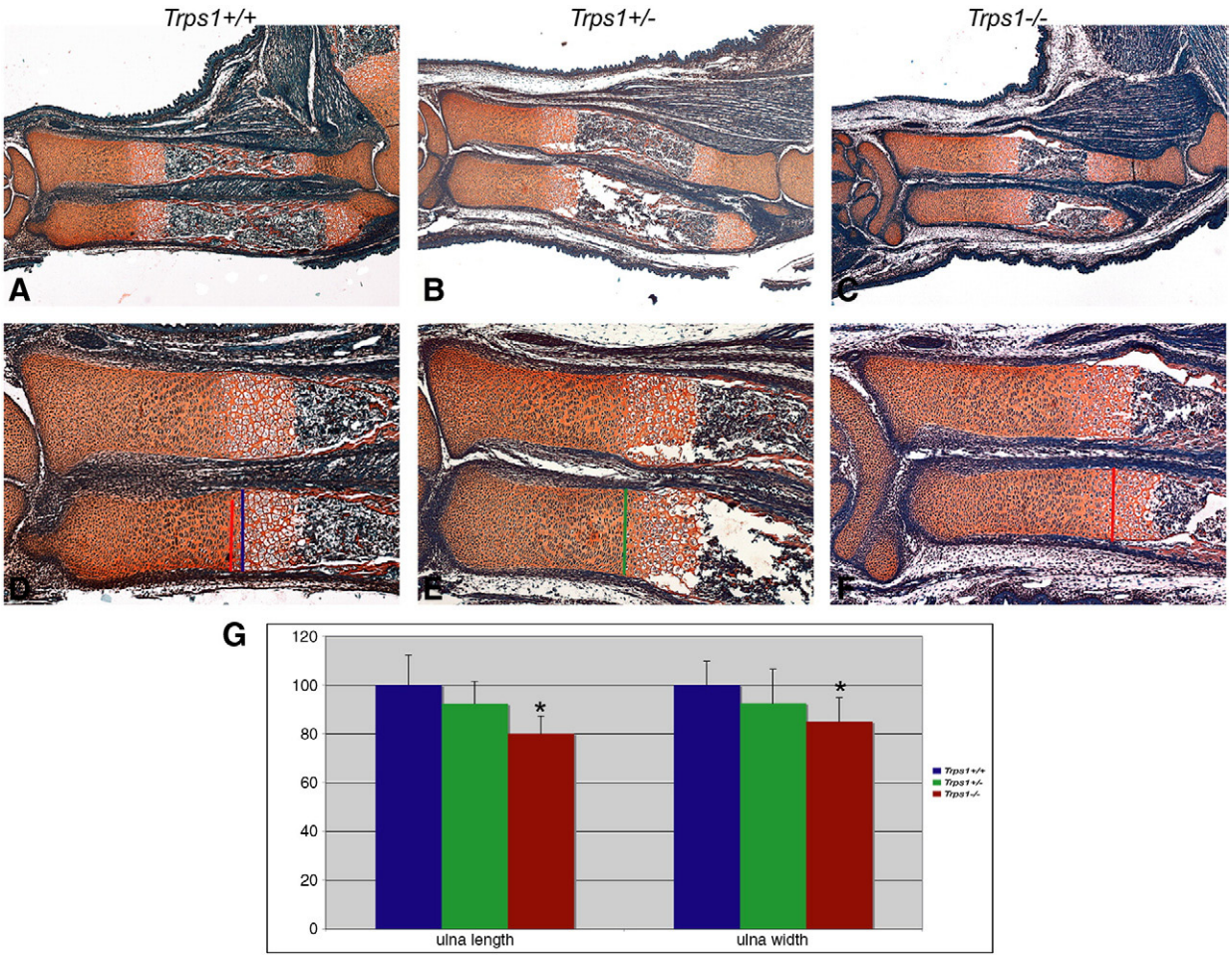


Fig. 1. Morphological analysis of endochondral ossification in *Trps1*$^{-/-}$ mice. Safranin–Weigert staining of E16.5 wild type (A, D), heterozygous (B, E) and *Trps1* deficient mice revealed shorter and smaller bones in *Trps1*$^{-/-}$ mice (C,F: red line) compared to wild type littermates (A, D: blue line). *Trps1*$^{+/-}$ mice have an intermediate phenotype with less pronounced shortening of the limbs (B, E: green line). Upper panel: 50× magnification, lower panel: 100× magnification. G: Statistical analysis of total ulna length and width. In E16.5 *Trps1*$^{-/-}$ mice the ulna is reduced to 80% of wild type length. Similarly, the width is reduced to 82% of that of wild type. *p-value of ulna length: 0.018, *p-value of ulna width: 0.00047 (n=5).

and 82% in diameter (Fig. 1G). Bone formation is delayed resulting in a reduced ossified zone (Fig. 1C). Interestingly, *Trps1*$^{+/-}$ heterozygous mice display an intermediate phenotype with a slightly reduced bone length and width if compared to littermates (Figs. 1B, E). Due to phenotypic variation these changes are, however, not statistically significant between litters.

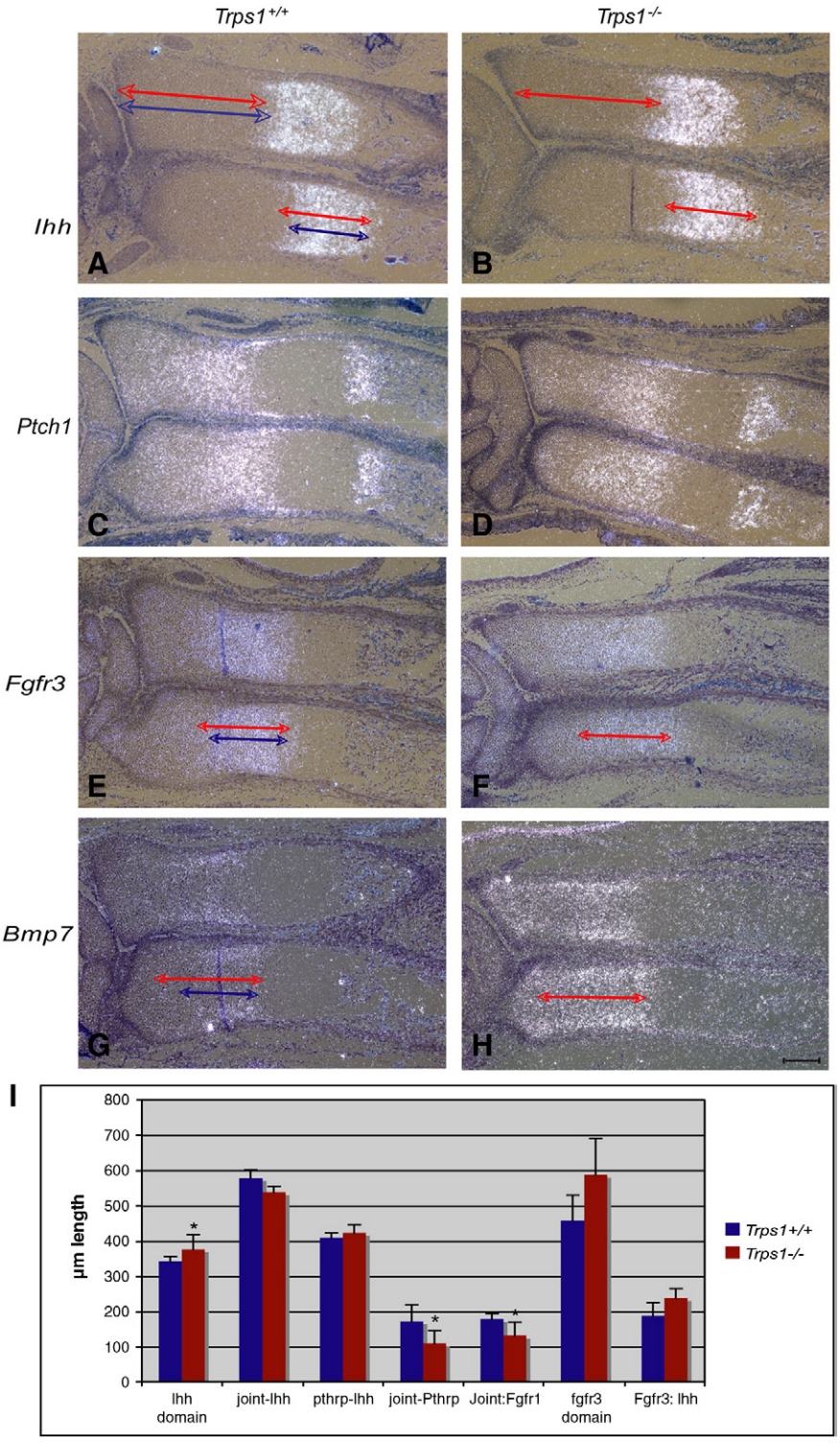


Fig. 2. Trps1 regulates the differentiation of proliferating chondrocytes. *In situ* hybridization on E16.5 forelimb sections of wild type (A, C, E, G, blue arrows) and *Trps1*$^{-/-}$ mice (B, D, F, H, red arrows) with antisense riboprobes for *Ihh* (A, B), *Ptch1* (C, D), *Fgfr3* (E, F) and *Bmp7* (G, H) revealed an expanded *Ihh* expression domain of *Ihh* in prehypertrophic chondrocytes of *Trps1*$^{-/-}$ mice (B), while the *Ptch1* expression is not altered in *Trps1*$^{-/-}$ mice (D). The zones of *Fgfr3* and *Bmp7* expression are enlarged in columnar and prehypertrophic chondrocytes of *Trps1*$^{-/-}$ mutants (F, G) compared to wild type mice (E). 100× magnification. I: Relative size of expression domains in *Trps1*$^{-/-}$ (red bars) and wild type mice (blue bars). The expression domains of *Fgfr1* and *PTHrP* are significantly reduced in distal chondrocytes of *Trps1*$^{-/-}$ mice, while the distance between *Ihh* and *PTHrP*, demarcating columnar chondrocytes, is not significantly altered. The zone of *Ihh* expression in prehypertrophic chondrocytes is expanded. Similarly, the zone of prehypertrophic chondrocytes expressing *Ihh* and *Fgfr3* is enlarged. Values are given in µm length (*$p<0.05$; $n=5$).

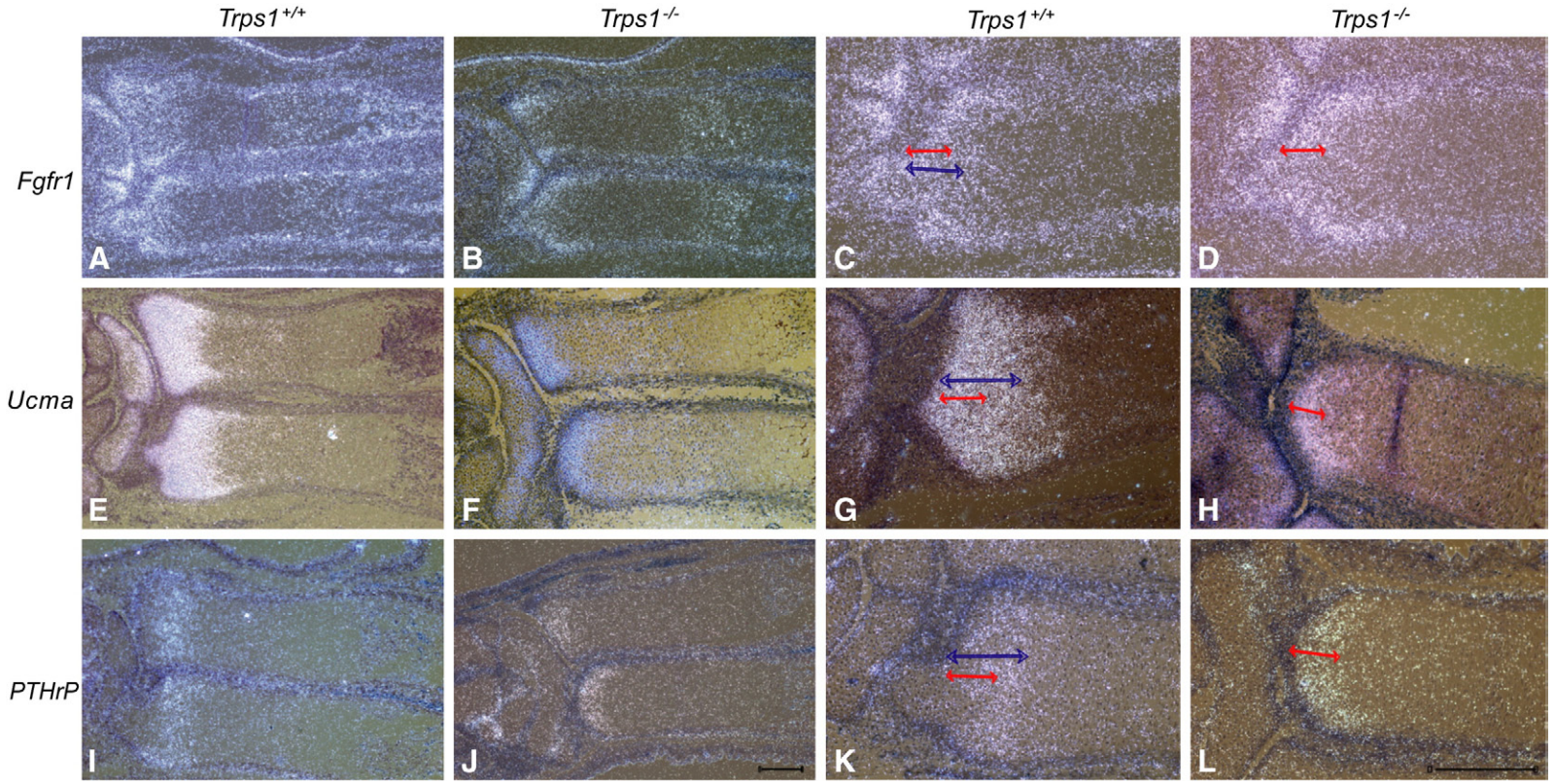


Fig. 3. The zone of distal chondrocytes is reduced in *Trps1*$^{-/-}$ mice. *In situ* hybridization on E16.5 forelimb sections of wild type (A, C, E, G, I, K, blue arrows) and *Trps1*$^{-/-}$ mice (B, D, F, H, J, L red arrows) with antisense riboprobes for *Fgfr1* (A–D), *Ucma* (E–G) and *PTHrP* (H–L). The zone of distal chondrocytes expressing *Fgfr1* (A–D) and *Ucma* (E–G) is reduced in *Trps1*$^{-/-}$ mutants (D, H) compared to wild type mice (C, G). The domain of *PTHrP* expression is restricted to the most distal cells in *Trps1*$^{-/-}$ mice (J, L), whereas in wild type ulna and radius the most distal cells have ceased to express *PTHrP* (I,K). A, B, E, F, I, J: 100× magnification, C, D,G, H, K, L: 200× magnification.

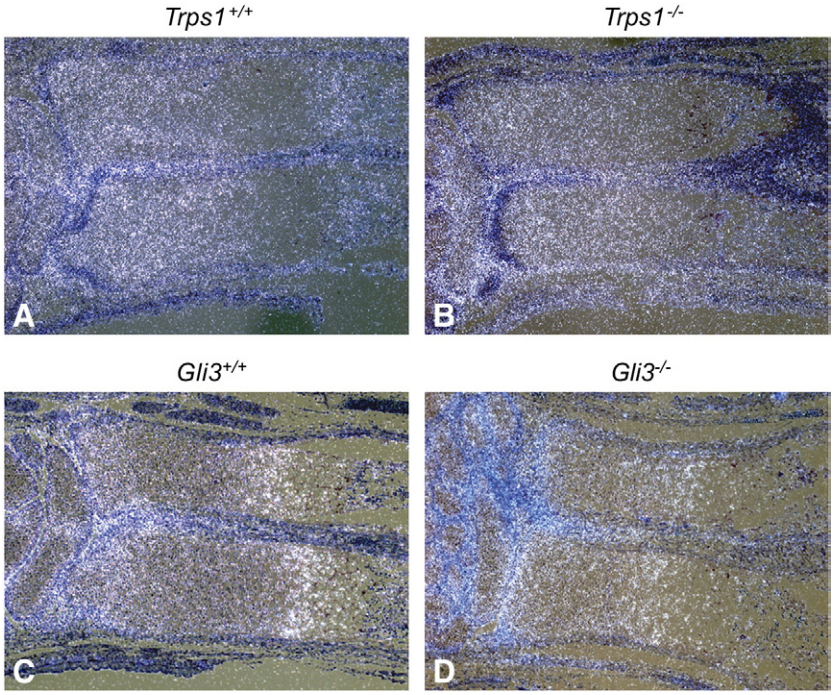


Fig. 4. Trps1 and Gli3 do not mutually regulate their transcription. *In situ* hybridization on E16.5 forelimb sections of wild type (A, C), *Trps1*$^{-/-}$ (B) and *Gli3*$^{-/-}$ mice (D) revealed no differences in *Gli3* mRNA expression (A, B) between *Trps1*$^{-/-}$ (B) and wild type mice (A). Vice versa, *Trps1* mRNA expression is not altered in *Gli3*$^{-/-}$ (D) compared to wild type mice (C). 100× magnification.

Trps1 regulates the size of the zone of distal chondrocytes

Based on morphological analysis of Safranin–Weigert stained limb sections, the zone of proliferating chondrocytes seems to be slightly enlarged in *Trps1*$^{-/-}$ mice (Fig. 1F). In agreement with other reports, we found the proliferation rate of columnar chondrocytes in *Trps1*$^{-/-}$ mice to be reduced to 80% of wild type levels (Figs. 6A, C and Napierala et al., 2008; Suemoto et al., 2007). As a reduced proliferation rate is frequently associated with a reduced region of proliferating chondrocytes, we investigated the size of this region using molecular markers. The transition from proliferating into hypertrophic chondrocytes is demarcated by the onset of *Ihh* expression. The distance from the joint to the distal limit of the *Ihh* expression domain therefore defines the region of proliferating chondrocytes (MacLean and Kronenberg, 2005). We found a slight expansion of the zone of *Ihh* expression. Nevertheless, measurement of the distance between *Ihh* and the joint region demonstrated that the domain of proliferating chondrocytes is slightly, but not significantly, reduced in *Trps1*$^{-/-}$ mutants (Figs. 2A, B, I).

The zone of proliferating chondrocytes can further be subdivided into distal chondrocytes, which express *Fibroblast growth factor receptor 1 (Fgfr1)* and Unique cartilage matrix-associated protein (*Ucma*), and columnar chondrocytes, which are characterized by the expression of *Bone Morphogenetic Protein* 7 (*Bmp7*), *Fgfr3* and the lack of *Ihh* expression (Minina et al., 2005; Tagariello et al., 2008). In *Trps1*$^{-/-}$ mutants the expression domain of *Fgfr1* (Figs. 3A–D) and *Ucma* (Figs. 3E–H) is significantly reduced to 84% compared to that of wild type mice (Fig. 2I). Trps1 seems thus to play an important role in expanding the pool of distal chondrocytes. Since the zone of distal chondrocytes is reduced in *Trps1*$^{-/-}$ mutants, we next asked if the expression of *PTHrP* is similarly altered. In E14.5 wild type mice, *PTHrP* is expressed in the most distal chondrocytes. At E16.5 the *PTHrP* expression domain is shifted from the distal end towards more central chondrocytes, releasing a small zone of chondrocytes directly at the joint that lacks *PTHrP* expression (Figs. 3I–L). In contrast to wild type mice, *in situ* hybridization of E16.5 *Trps1*$^{-/-}$ mutants revealed a restriction of the *PTHrP* expression domain to a small zone of distal chondrocytes, directly flanking the joint, indicating a role of Trps1 in locating the expression domain of *PTHrP*.

Analysis of *Fgfr3* (Figs. 2E, F) and *Bmp7* (Figs. 2E, G, H) expression revealed slightly enlarged domains (Fig. 2I) indicating either an increased region of columnar cells or a disturbed differentiation of proliferating into hypertrophic cells. To distinguish between these possibilities we determined the pool of columnar chondrocytes by calculating the distance between *PTHrP* (in distal cells) and *Ihh* (in hypertrophic cells) and found it to be slightly, but not significantly, increased (Fig. 2I). Instead the region of cells that are undergoing the early steps of hypertrophic differentiation and express *Fgfr3* and *Ihh* was expanded, pointing to a disturbed differentiation of columnar into hypertrophic chondrocytes.

In summary, Trps1 seems to execute multiple functions in proliferating chondrocytes, expanding the region of distal chondrocytes, activating proliferation in columnar cells and supporting the differentiation of columnar into hypertrophic chondrocytes.

Trps1 and Gli3 do not mutually regulate their transcription

A reduced zone of distal chondrocytes and a restriction of the *PTHrP* expression domain have been reported in *Gli3*$^{-/-}$ mutants (Koziel et al. 2005). To investigate the possibility that *Trps1* genetically interacts with Ihh/Gli3 signaling, we analyzed the expression of *Gli3* in *Trps1*$^{-/-}$ mice and of *Trps1* in the mouse mutant *Extra toes*, which carries an inactivated allele of *Gli3* (*Gli3*^{Xtj}; here designated *Gli3*$^{-/-}$, Hui and Joyner, 1993). We did not detect altered mRNA expression in either mutant (Fig. 4) indicating that these transcription factors do not mutually regulate their transcription.

Trps1 and Ihh/Gli3 signaling maintain distal chondrocytes

To further investigate a functional interaction between Gli3 and Trps1 we generated *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants and compared their phenotype to that of either single mutant. Molecular analysis of

chondrocyte markers revealed that the zone of distal chondrocytes, expressing *Fgfr1* (data not shown) and *Ucma*, is similarly reduced in *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ mutants as in either single mutant (Figs. 5M–O, S–V). Correspondingly, the expression domain of *PTHrP* in *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants remains restricted to the most distal cells similar to that in *Trps1*$^{-/-}$ and *Gli3*$^{-/-}$ single mutants (Figs. 5G–J). *Col2-Ihh* mice, that overexpress *Ihh* under the *Collagen Type 2* promoter (Long et al., 2001), have been reported to display a reduced domain of distal chondrocytes and a restricted domain of *PTHrP* expression (Kobayashi et al., 2005; Koziel et al., 2005). Analysis of *Ucma* and *PTHrP* expression in *Trps1*$^{-/-}$;*Col2-Ihh* mutants revealed the zone of distal chondrocytes to be similarly restricted to the joint region as in *Trps1*$^{-/-}$ mutants (Figs. 5J–L, P–R, V–X). Analysis of these mutants was, however, complicated by the fact that carpal bones are frequently fused to the distal ends of ulna and radius making it difficult to define the borders of individual bones (Fig. 5, blue lines in K, Q, W, L, R, X). In summary, the similarity of the investigated phenotypes supports a cooperative action of Trps1 and Gli3 in regulating the pool of distal chondrocytes.

Trps1 activates chondrocyte proliferation in parallel to Ihh signaling

Morphological analysis of *Gli3*$^{-/-}$ mutants revealed a slightly reduced length, but a normal width of the ulna (Fig. 5B), whereas *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ mice (Fig. 5C) resemble the reduced bone length and diameter of *Trps1*$^{-/-}$ mutants (Fig. 5D). In contrast, the size of the skeletal elements of *Trps1*$^{-/-}$;*Col2-Ihh* mutants (Fig. 5E) is increased compared to that of *Trps1*$^{-/-}$ mutants (Fig. 5D), but decreased compared to *Col2-Ihh* mice (Fig. 5F). These changes prompted us to investigate differences in chondrocyte proliferation. As shown before, Trps1 acts as an activator of proliferation (Napierala et al., 2008; Suemoto et al., 2007). *Ihh* overexpressing mice display an increased proliferation rate (Fig. 6D, Long et al., 2001) while *Gli3*$^{-/-}$ mutants showed no obvious alterations in proliferation (Fig. 6C). Nevertheless, loss of Gli3 is sufficient to restore the severely reduced proliferation rate of *Ihh*$^{-/-}$ mice identifying Gli3 as a repressor of proliferation (Hilton et al., 2005; Koziel et al., 2005). Analysis of compound mutants revealed that loss of Gli3 does not increase chondrocyte proliferation in *Trps1*$^{-/-}$ mutants (Figs. 6A, C). In contrast, the proliferation rate in *Trps1*$^{-/-}$;*Col2-Ihh* mice is increased compared to that of *Trps1*$^{-/-}$ mutants (Figs. 6B, D), but does not reach the rate of *Col2-Ihh* mice. These data strongly indicate that loss of Gli3 repressor function is not sufficient to overcome the reduced proliferation rate in *Trps1*$^{-/-}$ mice. Instead, a direct activating function of either Gli3 or Gli2 seems to be required to increase chondrocyte proliferation in *Trps1*$^{-/-}$ mutants.

The transcription factors Trps1 and Gli3 form stable complexes in vitro and in vivo

As Trps1 and Gli3 seem to act at similar steps during chondrocyte differentiation we analyzed a potential interaction of these transcription factors on protein level. First, Cos7 cells were transiently transfected with plasmids encoding human FLAG-tagged TRPS1 (hTRPS1) and myc-tagged hGLI3A and hGLI3R proteins. Expression was monitored on western blot with a monoclonal α-c-myc and a polyclonal α-Trps1 antibody (Fig. 7B and Sup. Figs. 2A, B). After immunoprecipitation with M2 anti-FLAG agarose or the polyclonal α-TRPS1 antibody we could detect hTRPS1-FLAG fusion protein on western blot (Fig. 7C). After co-transfection of the respective proteins the anti-FLAG agarose or the polyclonal α-TRPS1 antibody precipitates stable complexes of hTRPS1 and hGLI3A but not with Gli3R (Figs. 7D, E). In the reverse experiment, the α-c-myc antibody detects complexes of hTRPS1 and hGLI3A, whereas no complexes can be detected with hGLI3R (Sup. Fig. 2C). Interestingly in both experiment, only the GLI3 activator, but not the Gli3 repressor form was found to co-precipitate with hTRPS1.

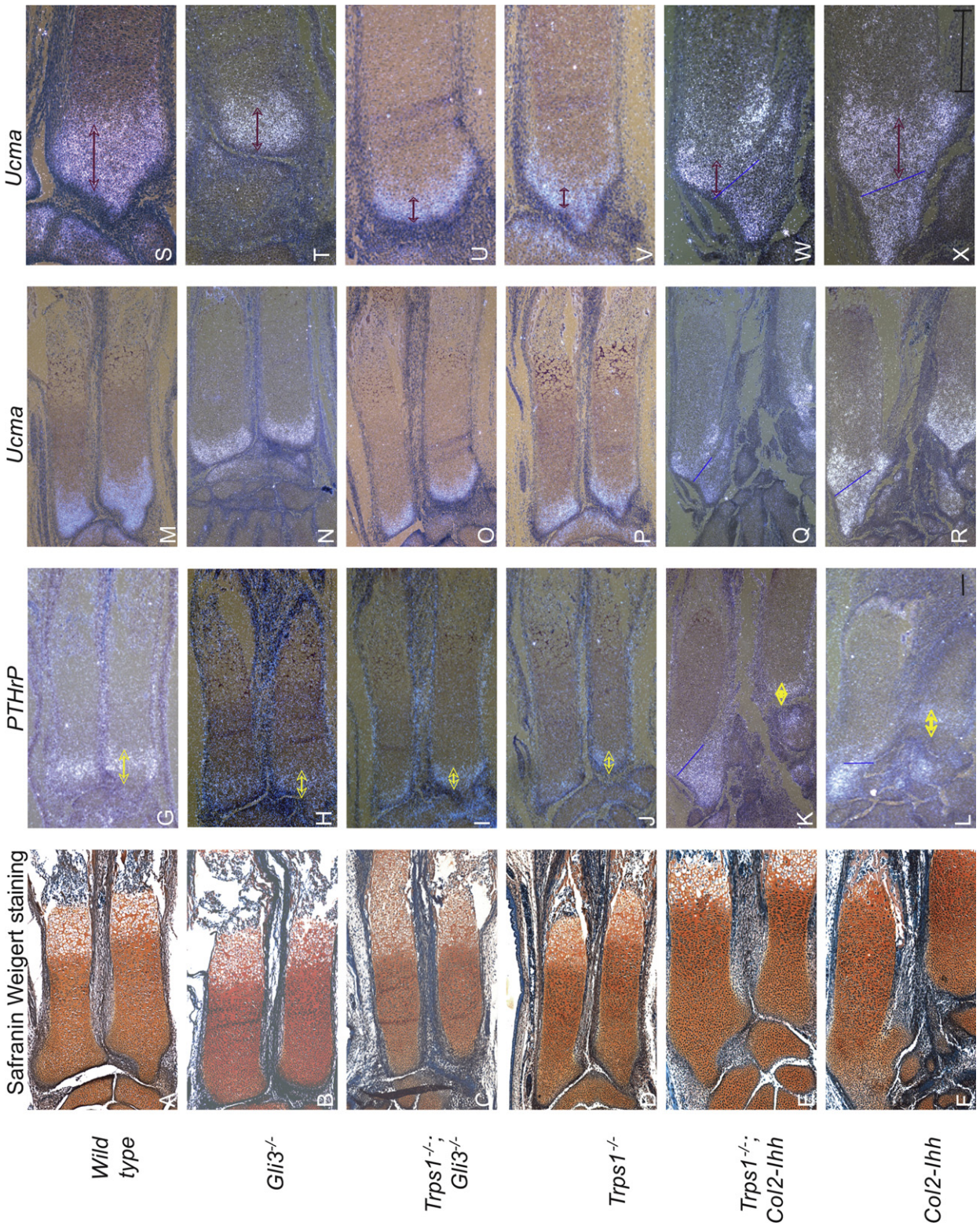
To confirm that Trps1 and Gli3 interact *in vivo* we analyzed protein extracts of E16.5 mouse limbs. By western blotting we detected a band at 160 kDa for Trps1 and two bands at 190 kDa and 90 kDa representing Gli3A and Gli3R, respectively (Figs. 8A, B: input). After co-immunoprecipitations with a Gli3 specific antibody we could identify Trps1 as a Gli3-binding protein *in vivo* (Fig. 8D). To verify the specific interaction of Trps1 with Gli3 protein, we performed co-immunoprecipitation in wild type and *Gli3*$^{-/-}$ mice, which lack the Gli3 activator and repressor form (Sup. Fig. 2E, Litingtung et al., 2002). In *Gli3*$^{-/-}$ mice, Trps1 cannot be precipitated while in wild type mice, the α-Gli3 antibody co-precipitates Trps1 (Sup. Fig. 2F). As the Gli3 specific antibody does not distinguish between the activator and repressor form of Gli3 (Wang et al., 2000; Hu et al., 2006) we next used the α-Trps1 antibody for immunoprecipitation and found an interaction of Trps1 with the 190 kDa Gli3A protein only, whereas the 90 kDa Gli3R protein was not bound by Trps1 (Fig. 8B). This was especially surprising as in relation to Gli3R only limited amounts of Gli3A could be detected in limb protein extracts (Fig. 8B: input, Wang et al., 2000). These data strongly indicate a specific interaction of Trps1 and Gli3A, while Gli3R does not seem to bind to Trps1 *in vivo*. These experiments also identify the C-terminal part of Gli3 as the domain interacting with Trps1.

The Gli3 transactivator domain interacts with the central zinc finger region of Trps1

In order to characterize the domain of Trps1 that interacts with Gli3 we first analyzed binding of Trps1 to Gli3 in *Trps1*$^{-/-}$ mice. In these mice an in frame deletion of 41 aa (aa 888 to 928) encoding exon 4 results in a mutant protein lacking the GATA-type zinc finger (Fig. 8C, Malik et al., 2002). Immunoprecipitation with α–Gli3 antibody revealed similar binding of mutant and wild type Trps1 protein to Gli3 (Fig. 8D) indicating that the DNA-binding GATA domain is not essential for the interaction.

To further confine the domain of Trps1 that interacts with Gli3, we analyzed binding of different subfragments of Trps1 (Figs. 7A and 8F, Kaiser et al., 2003a,b) in GST pull-down assays. For Gli3 two overlapping parts of the C-terminal activator domain of hGLI3 were cloned and purified as GST fusion proteins (Figs. 7A and 8E): GLI3A-TA1 encoding the complete C-terminal region (aa 745–1580) and GLI3A-TA2 representing a shorter C-terminal part (aa 827–1580). In GST pull-down assays, we could not detect significant binding of TRPS1-F1 (aa 1185–1281), which consists of the Ikaros-type zinc finger domain to either of the GLI3A-TA fragments (Fig. 8G), excluding this protein binding domain as the main interacting region. The two overlapping C-terminal fragments TRPS1-F5 (aa 635–1281) and TRPS1-F6 (aa 635–1184) were retained in the pull-down assays with

Fig. 5. Trps1 and Ihh/Gli3 signaling maintain distal chondrocytes. Morphological analysis of E16.5 forelimb sections after Safranin–Weigert staining (A–F) revealed a reduced bone width in *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ double mutants (C) similar to that in *Trps1*$^{-/-}$ mutants (D). *Gli3*$^{-/-}$ mice (B) display a reduced bone length whereas the ulna width is not altered. The width of the skeletal elements of *Trps1*$^{-/-}$;*Col2-Ihh* mice (E) is increased compared to *Trps1*$^{-/-}$ mutants (D), but decreased compared to *Col2-Ihh* mice (F). *In situ* hybridization with a *PTHrP* riboprobe (G–L) detects a restricted domain of *PTHrP* expression (yellow arrows) in *Gli3*$^{-/-}$ (H), *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ (I), *Trps1*$^{-/-}$ (J), *Trps1*$^{-/-}$;*Col2-Ihh* (K) and *Col2-Ihh* mice (L) that is not shifted towards the central zone of the skeletal elements. The *Ucma* expression domain (M–X, red arrows) in distal chondrocytes is reduced in *Gli3*$^{-/-}$ (N,T), *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ (O, U), *Trps1*$^{-/-}$ (P,V), *Trps1*$^{-/-}$;*Col2-Ihh* (Q, W) and *Col2-Ihh* mutants (R, X), compared to wild type mice (M,S), indicating a reduced number of distal chondrocytes. Blue lines demarcate the border of individual skeletal elements, which are fused in *Trps1*$^{-/-}$;*Col2-Ihh* (K, Q, W) and *Col2-Ihh* mutants (L, R, X). A–R: 100× magnification, S–U: 200× magnification.


Safranin Weigert staining
PTHrP
Ucma
Ucma
Wild type
Gli3-/-
Trps1-/-; Gli3-/-
Trps1-/-
Trps1-/-; Col2-Ihh
Col2-Ihh
A
B
C
D
E
F
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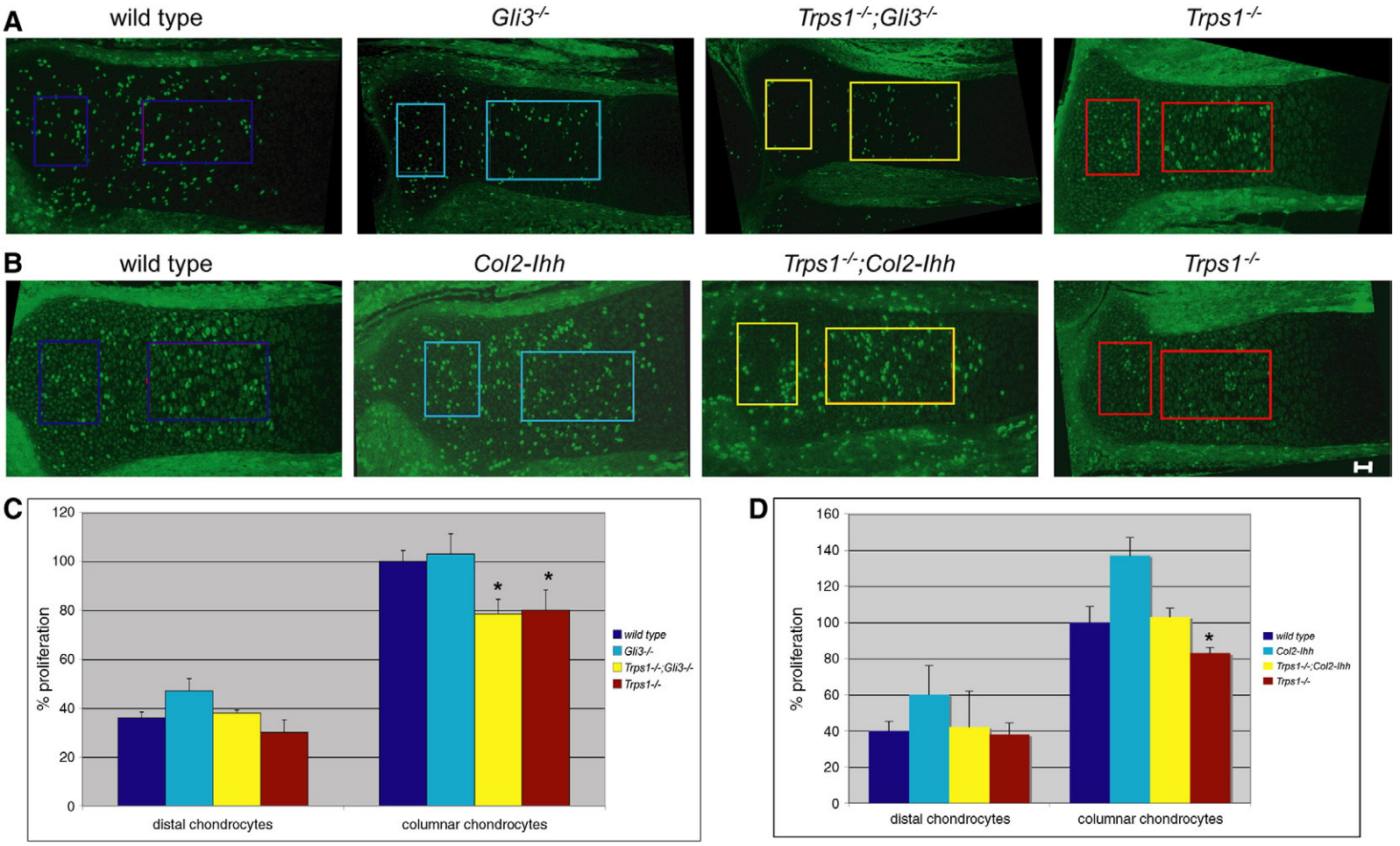


Fig. 6. Trps1 and Ihh signaling activate chondrocyte proliferation. (A) BrdU labeling of chondrocytes in E16.5 forelimb sections revealed a reduced proliferation rate in columnar chondrocytes of *Trps1*$^{-/-}$ (red) and *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ (yellow) mutants, while in *Gli3*$^{-/-}$ mutants (light blue) the proliferation rate is not significantly altered compared to wild type (dark blue). (B) Overexpression of *Ihh* (light blue) increases the proliferation rate in columnar chondrocytes compared to wild type (dark blue) and *Trps1*$^{-/-}$ mice (red). In *Trps1*$^{-/-}$;*Col2-Ihh* compound mutants (yellow) the proliferation rate is increased compared to *Trps1*$^{-/-}$ mutants (red), but decreased in relation to *Col2-Ihh* mice (light blue). (C and D) Statistical analysis of chondrocyte proliferation rates depicted in A and B, respectively. (n=4, *p<0.05). Scale bar: 10 μm.

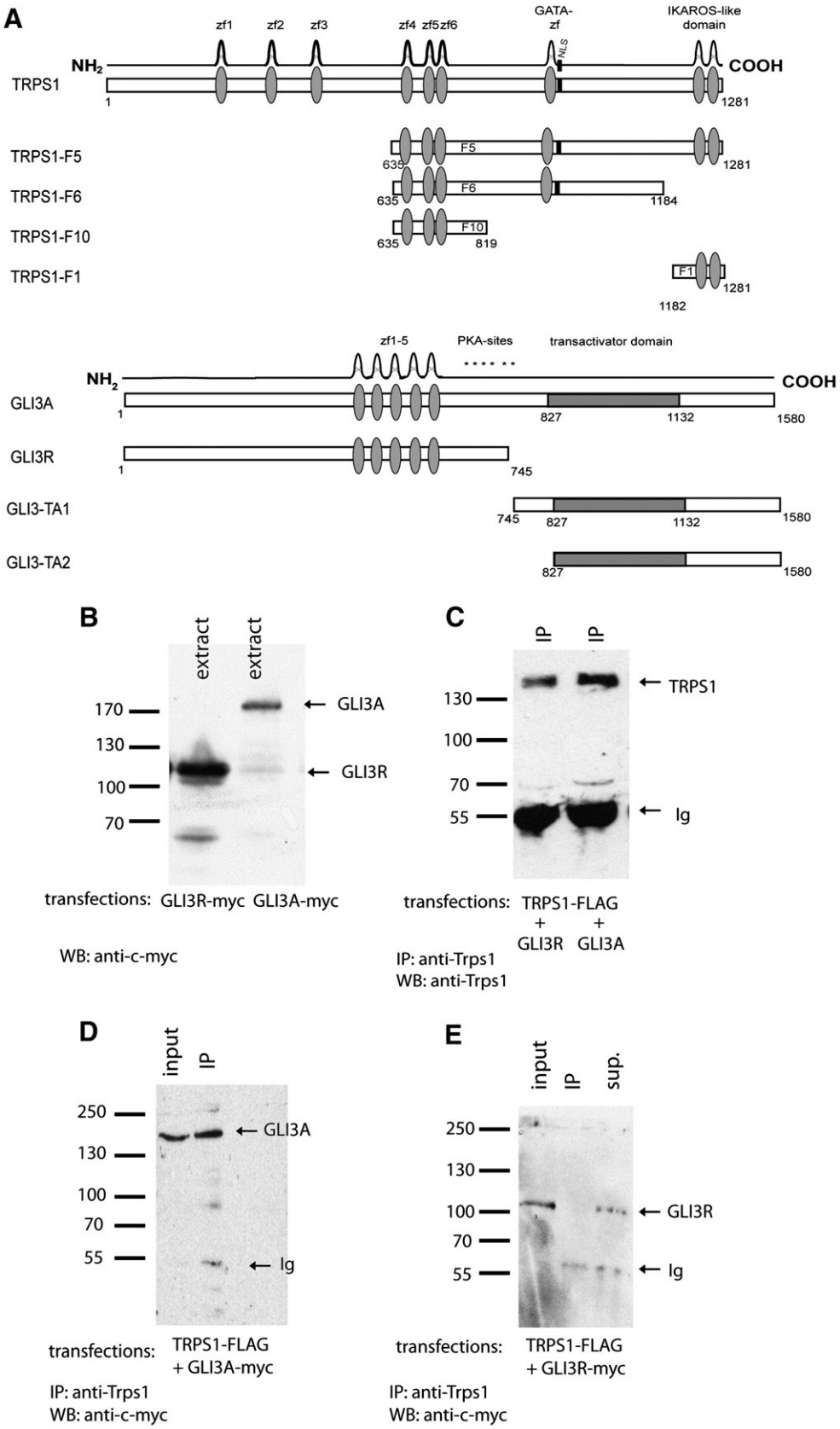


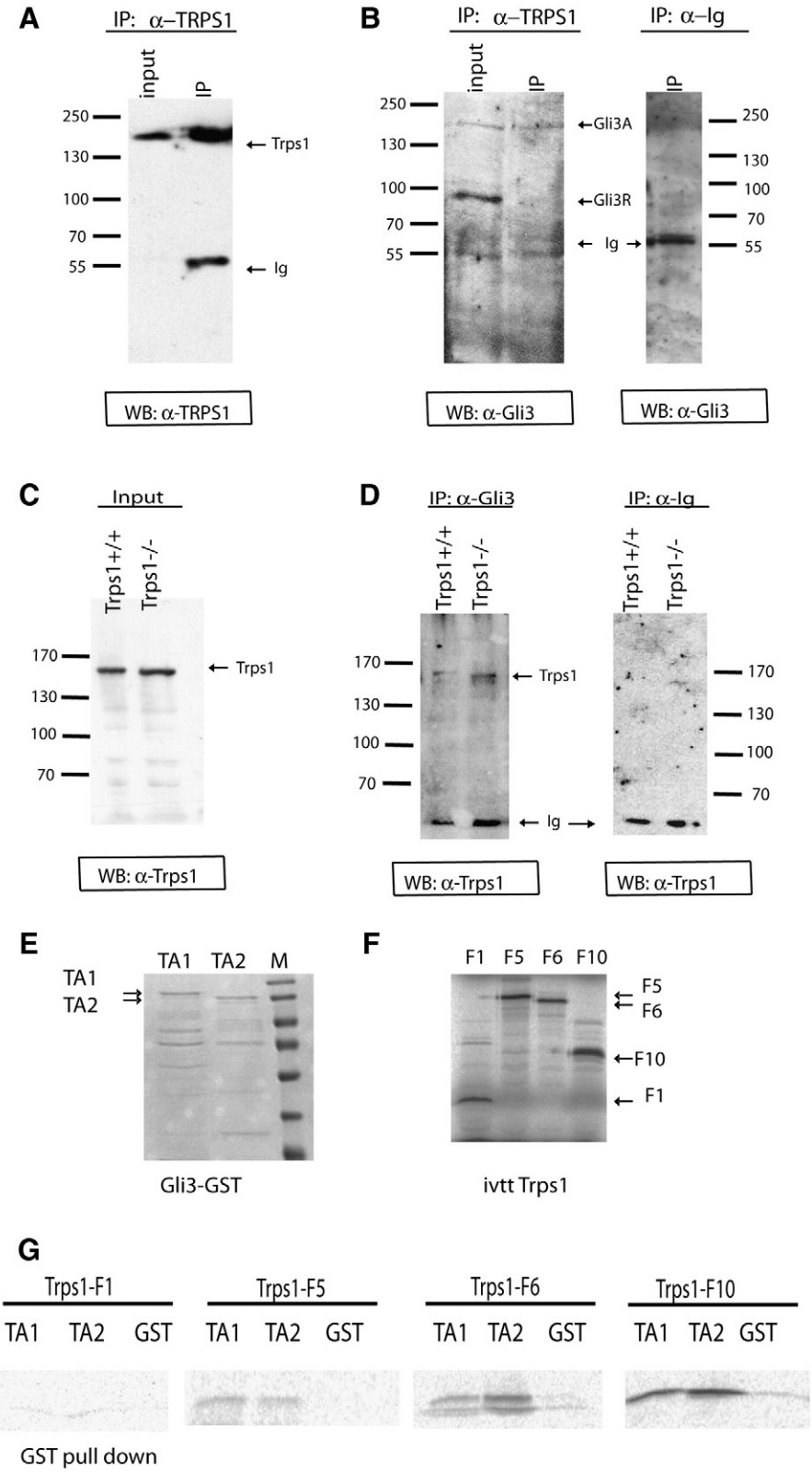
Fig. 7. TRPS1 selectively interacts with the GLI3 activator form. (A) Diagram of the expression constructs used in interaction studies of hGLI3 and hTRPS1. B: After overexpression in Cos7 cells, the c-myc specific antibody detects a band of 190 kDa for hGLI3A-myc and of 110 kDa for hGLI3R-myc on western blot. (C–E) Co-transfection of hTRPS1-Flag with hGLI3R-myc or hGLI3A-myc followed by co-immunoprecipitation with an α-TRPS1 or an α-FLAG antibody and Western Blot with the indicated antibodies. The α-TRPS1 and the α-FLAG antibodies detect hTRPS1-FLAG as a band at 160 kDa on western blot (C). The c-myc specific antibody detects a specific interaction of hGLI3A with hTRPS1 (D), whereas no interaction was observed with hGLI3R (E). Input: protein extracts used for immunoprecipitation, IP: immunoprecipitation, WB: western blot: aa: amino acids zf: zinc finger domain.

both GLI3A-TA fusion proteins (Fig. 8G). Stringent binding to GLI3A-TA1 and 2 was also observed for TRPS1-F10 (aa 635–819), containing zf 4–6 only (Fig. 8G). These experiments allowed us to define a stretch of 185 aa (aa 635–819) encoding the three predicted $C_2X_{12}H_2$ zinc finger motifs 4–6 as the critical GLI3A binding domain within the TRPS1 protein.

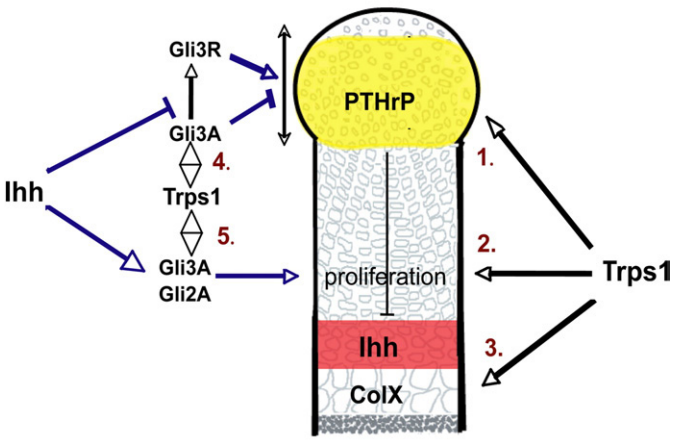
Discussion

Trps1 regulates differentiation in proliferating chondrocytes

The presented data have identified Trps1 as an essential transcription factor regulating chondrocyte proliferation and



A



B

Genotype	zone of distal chondrocytes	ulna length	ulna width	proliferation	rescued proliferation rate
Wild type	normal	normal	normal	normal	/
Trps1-/-	reduced	decreased	decreased	reduced	/
Gli3-/-	reduced	decreased	normal	normal	/
Trps1-/-;Gli3-/-	reduced	decreased	normal	reduced	no
Col2-Ihh	reduced	increased	increased	increased	/
Trps1-/-;Col2-Ihh	reduced	normal	normal	normal	yes

Fig. 9. Trps1 regulates different steps of chondrocyte differentiation in parallel to Ihh/Gli3 signaling (A) 1. In distal chondrocytes, Trps1 maintains distal chondrocytes and localizes the *PTHrP* expression domain. PTHrP signals through the growth plate to inhibit hypertrophic differentiation 2. In columnar chondrocytes, Trps1 activates chondrocyte proliferation. 3. In hypertrophic chondrocytes, Trps1 supports the differentiation of *Ihh* expressing prehypertrophic chondrocytes (red) independent of Gli3. 4. Trps1 seems to regulate the pool of distal cells by inhibition of Gli3A. 5. During chondrocyte proliferation Trps1 might either inhibit Gli3A to allow Gli2 to preferentially activate proliferation. Alternatively Trps1 and Gli3 act in a complex to regulate proliferation. (B) Overview of the phenotypic differences between the analyzed single and compound mutants. Chondrocyte proliferation could only be rescued by activated Ihh signaling (*Trps1*$^{-/-}$;*Col2-Ihh* mice).

differentiation. In particular, we and others (Napierala et al., 2008; Suemoto et al., 2007) have demonstrated that Trps1 activates proliferation in columnar chondrocytes. Although in *Trps1*$^{-/-}$ mutants the proliferation rate is significantly reduced we found that the region of proliferating chondrocytes is only slightly reduced compared to wild type animals. This prompted us to analyze subpopulations of proliferating cells in more detail. We found that the region of distal chondrocytes expressing *PTHrP*, *Fgfr1* and *Ucma* is significantly reduced in *Trps1*$^{-/-}$ mutants, whereas the region of columnar chondrocytes demarcated by the distance between the *PTHrP* and *Ihh* expression domain is not significantly altered. Unlike our data, other studies described an increased region of proliferating cells in E18.5 femurs and newborn tibiae, possibly reflecting differences in age or in individual skeletal elements (Napierala et al., 2008; Suemoto et al., 2007). Like Napierala et al., we detected an increased region of *Ihh* expression and could demonstrate that the region of prehypertrophic cells that express *Fgfr3* and *Ihh* is increased in *Trps1*$^{-/-}$ mutants. Cells in this region appear columnar by morphology, likely contributing to the differences observed in the size of the proliferating region.

In summary, Trps1 seems to mediate different effects in proliferating chondrocytes, activating proliferation and differentiation of columnar chondrocytes and maintaining the population of distal cells (Fig. 9A). It is thus likely that cell type specific interaction partners define its function in distinct cell types.

Trps1 specifically interacts with the activator form of Gli3

As Trps1 and Ihh/Gli3 signaling regulate similar steps of chondrocyte differentiation, we investigated a potential physical interaction of Trps1 and Gli3 by co-immunoprecipitation. We identified stable complexes of Trps1 and Gli3 *in vitro* and *in vivo* in limb protein extracts. As outlined above, in absence of Hh signaling Gli3 is processed into a short N-terminal repressor, whereas Hh signaling stabilizes the full length Gli3 activator (Hilton et al., 2005; Low et al., 2008). In accordance with previous reports (Pan and Wang, 2007; Wang et al., 2000) we found higher levels of Gli3R than Gli3A in limb protein extracts. Nevertheless, by co-immunoprecipitation and GST pull-down experiments we detected a specific interaction of zinc fingers 4–6 of hTRPS1 with the transactivation domain of hGLI3. These experiments identify Trps1 as one of the first transcription factors that specifically binds to the activator form of Gli3. Until now, only the CREB binding protein CBP and the Mediator complex have been reported to specifically interact with the transactivation domain of Gli3, thereby releasing the transcriptional repression of Hh target genes (Zhou et al., 2006). If Trps1 is also recruited to these complexes or if Trps1 binding inhibits complex formation of Gli3 and CBP or Mediator, thereby preventing the activation of target genes, is an important question for future studies.

Similarly, only few proteins have been identified that interact with Trps1. The ring finger protein RNF4 binds to a domain between the GATA and the Ikaros zinc fingers and was shown to inhibit Trps1 mediated repression of transcription (Kaiser et al., 2003a). Recently, Runx2 has been described as a Trps1 interacting protein. The authors defined the Runt domain of Runx2 and the Ikaros domain of Trps1 as the interacting regions, whereas the other zinc fingers of Trps1 are not required for binding (Napierala et al., 2008). The Ikaros domain of Trps1 has also been reported to interact with the dynein light chain protein LC8a, which inhibits Trps1 repressor activity (Kaiser et al., 2003b). Interestingly, binding of Trps1 to LC8a can also be mediated by the domain containing zinc fingers 4–6 (aa 635–819) of Trps1 (Kaiser et al., 2003b). Here we have demonstrated that the same region is sufficient for binding to the Gli3 transactivation domain, emphasizing the functional significance of this zinc finger region as a protein binding motif within Trps1.

Fig. 8. A 185 aa domain of TRPS1 encoding three $C_2X_{12}H_2$ zinc finger motifs (zf 4–6) is sufficient to bind to the transactivator domain of GLI3. (A) Trps1 is detectable in limb protein extracts of E16.5 wild type mice (input) and after immunoprecipitation with a Trps1 specific antibody (IP). (B) On western blot, bands at 190 kDa for Gli3A and 90 kDa for Gli3R can be detected in protein extracts of E16.5wild type limbs (input), with Gli3R being expressed at higher levels than Gli3A. The α-Trps1 antibody specifically precipitates the 190 kDa Gli3A protein, while the 90 kDa Gli3R protein is not precipitated (IP). Immunoprecipitation with an Ig-antibody as control shows no retention of either Gli3A or Gli3R. (C) Similar amounts of Trps1 protein can be detected in limb extracts of wild type (*Trps1*$^{+/+}$) and *Trps1*$^{-/-}$ mice. In these mice an in frame deletion of 41 aa leads to the formation of a mutant protein lacking the GATA-type zinc finger. (D) After co-immunoprecipitation with a Gli3 specific antibody binding of Trps1 to Gli3 can be detected in *Trps1*$^{-/-}$ and wild type mice, while an unspecific Ig antibody did not precipitate Trps1. (E) Coomassie staining detects purified GST-tagged GLI3-TA1 (aa 745–1580) and GLI3-TA2 (aa 827–1580) after SDS-PAGE. (F) Radiography of *in vitro* translated, radiolabeled hTRPS1 fragments. (G) GST pull-down assay does not detect an interaction of TRPS1-F1 (aa 1185–1281) encoding the Ikaros-type zinc finger domain with either of the GLI3-TA constructs. TRPS1-F5 (aa 635–1281) and F6 (aa 635–1184) are retained by GLI3-TA1 and GLI3-TA2 GST-fusion protein. TRPS1-F10 (aa 635–819) representing zf 4–6 of hTRPS1 is sufficient to bind to GLI3-TA1 and Gli3-TA2 in pull-down experiments. Input: protein extracts used for immunoprecipitation, IP: immunoprecipitation, WB: western blot: TA: transactivator domain, zf: zinc finger domain.

Interaction of Trps1 and Ihh signaling in chondrocytes

As described above, the zone of distal chondrocytes is reduced in *Gli3*$^{-/-}$ and *Col2-Ihh* mutants. It was concluded that Gli3R function is required to expand the zone of distal chondrocytes whereas Gli3A reduces the zone of distal cells (Koziel et al., 2005). By analyzing *Trps1*$^{-/-}$, *Trps1*$^{-/-}$;*Gli3*$^{-/-}$ and *Trps1*$^{-/-}$;*Col2-Ihh* mice we have identified Trps1 as a second transcription factor required to maintain distal cells in parallel to Ihh/Gli3 signaling. Like Gli3R, Trps1 seems to antagonize Gli3A function. The interaction of Trps1 and Gli3A identified in this study might thus inhibit residual Gli3A activity in distal cells or turn Gli3A into a functional repressor. A similar role in modulating Gli3 activity has been described for HoxD12, which upon binding to Gli3R converts the repressor into an activator of transcription (Chen et al., 2004). Alternatively, binding of Trps1 and Gli3A to yet unknown target promoters, might be essential for the function of the complex.

In addition to regulating the domain of distal cells Trps1 and Ihh signals both activate chondrocyte proliferation. Loss of *Gli3* has no obvious effect on chondrocyte proliferation. However, in absence of Ihh signals Gli3 acts as a strong repressor of proliferation (Hilton et al., 2005; Koziel et al., 2005). In contrast to *Ihh*$^{-/-}$;*Gli3*$^{-/-}$ mutants, deletion of *Gli3* is not sufficient to overcome the reduced proliferation rate in *Trps1*$^{-/-}$ mutants, whereas overexpression of *Ihh* can partially rescue the proliferation rate. Besides demonstrating the cooperative role of Trps1 and Ihh signaling these data strongly indicate that an increased activator function of Gli3, or possibly Gli2, is necessary to induce chondrocyte proliferation in the absence of Trps1. This is supported by recent findings of Mau et al., which identify Gli2 as activator of chondrocyte proliferation downstream of Ihh (Mau et al., 2007) likely antagonizing the repressor function of Gli3. In proliferating cells Trps1 seems thus to exert its activating function, at least in part, by stabilizing the activator form of Gli3. If Trps1 also interacts with Gli2, which contains at least two regions of high homology to Gli3 in the C-terminal transactivation domain, will be of high interest for further studies.

As a third function in proliferating chondrocytes Trps1 seems to support the differentiation of proliferating into hypertrophic cells. This step is also regulated by Runx2, which binds to and is inactivated by Trps1 (Napierala et al., 2008). Trps1 might thus inhibit Runx2 function in late proliferating cells thereby sharpening the border between proliferating and hypertrophic chondrocytes. In these cells, Trps1, Runx2 and Gli3 might interact in a complex of higher order. Alternatively, binding of Gli3 to Trps1 might be antagonized by Runx2, thereby facilitating the switch into hypertrophic chondrocytes.

Our data reveal specific functions of Trps1 in different cell types likely by interacting with different subsets of co-factors. Trps1 can bind to DNA by its GATA-type zinc finger and contains at least three protein binding domains. It is thus tempting to hypothesize that it acts as an adapter protein tethering cell type specific subsets of transcription factors to distinct promoter regions. Differentiation dependent modulations in complex composition might switch the effect of Trps1/Gli3A from repressor to activator function. In addition to interacting in a complex, the overlapping but distinct phenotypes of *Gli3*$^{-/-}$ and *Trps1*$^{-/-}$ mutants imply additional independent roles of both transcription factors in distal and proliferating chondrocytes.

Interestingly Trps1 and Gli3 are expressed in overlapping domains in many organs including lung and gut mesenchym, kidney stroma, testis and distinct domains of the brain (Kunath et al., 2002; Mullor and Ruiz i Altaba, 2002). Strikingly, strong overlapping expression of *Trps1*, *Runx2* and components of the Sonic hedgehog (Shh) signaling pathway is found in the dermal papilla of hair follicles and mutations of *Trps1* or *Runx2* or impaired Shh signaling lead to defects in hair development (Fantauzzo et al., 2008a; Glotzer et al., 2008; Oro and Higgins, 2003). Similar to our data, recent studies on Trps1 function during hair formation demonstrated that, depending on the cellular context, Trps1 can act as a transcriptional activator or repressor (Fantauzzo et al., 2008b). It is thus intriguing to speculate that the physical interaction of Trps1 and Gli3 identified in this study is also critical for the differentiation of other organs.

TRPS1 function and the human phenotype

Patients with TRPS are characterized by short stature, delayed formation, but premature closure of the growth plate, and distinctive cone-shaped epiphyses (Momeni et al., 2000). Genotype-phenotype correlations revealed that patients carrying missense mutations in the GATA-type zinc finger develop the most severe skeletal malformations, while null mutations or in-frame deletion of the GATA zinc finger lead to a milder disease phenotype (Ludecke et al., 2001). The *Trps1*$^{-/-}$ mice investigated in this study harbor an in-frame deletion of the GATA-type zinc finger (Malik et al., 2001) and, similar as the mice published by Suemoto et al. (2007), represent a model for the milder variant TRPS Type I. Our investigation of *Trps1*$^{-/-}$ mice revealed a function of Trps1 in regulating the zone of distal cells. After birth, the secondary ossification centers form within theses cells, thereby separating the pool of distal cells into two subpopulations: Articular chondrocytes adjacent to the joint and resting chondrocytes at the distal zone of the growth plate (Chen et al., 2006). Reduced Trps1 expression in TRPS patients may thus cause a reduction in the pool of distal cells and subsequently lead to the observed delay in the development of secondary ossification centers. Moreover, resting chondrocytes have been proposed to harbor undifferentiated, stem cell like' cells that replenish the proliferating zone (Abad et al., 2002). Reduced numbers of these undifferentiated chondrocytes might therefore enhance the proliferation defect and lead to premature growth plate closure and the characteristically misshaped epiphyses.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.01.012.

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