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Gli2 and p53 Cooperate to Regulate IGFBP-3-Mediated Chondrocyte Apoptosis in the Progression from Benign to Malignant Cartilage Tumors

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SUMMARY

Clinical evidence suggests that benign cartilage lesions can progress to malignant chondrosarcoma, but the molecular events in this progression are unknown. Mice that develop benign cartilage lesions due to overexpression of Gli2 in chondrocytes developed lesions similar to chondrosarcomas when they were also deficient in p53. Gli2 overexpression and p53 deficiency had opposing effects on chondrocyte differentiation, but had additive effects negatively regulating apoptosis. Regulation of *Igfbp3* expression and insulin-like growth factor (IGF) signaling by Gli and p53 integrated their effect on apoptosis. Treatment of human chondrosarcomas or fetal mouse limb explants with IGFBP3 or by blocking IGF increased the apoptosis rate, and mice expressing Gli2 developed substantially fewer tumors when they were also deficient for *Igf2*. IGF signaling-meditated apoptosis regulates the progression to malignant chondrosarcoma.

INTRODUCTION

The progression of neoplasia from a benign lesion to malignancy is thought to require the progressive accumulation of mutations deregulating cell growth control, apoptosis, and DNA stability. Although this progression is identified in tumors of epithelial origin, it has not been demonstrated in mesenchymal tumors, in part because benign precursor lesions for most sarcomas are not clearly identified. This is not the case for enchondromas, which are benign cartilaginous tumors located in the metaphysis of bone. Enchondromas can occur as isolated or multiple lesions and can progress to malignant chondrosarcoma, an event that occurs at a higher rate in multifocal diseases. Although the factors implicated in the progression to chondrosarcomas are poorly understood, cytogenetic and mutational analysis of tumor suppressor genes identified mutations or cytogenetic anomalies in the p53 gene in roughly a third of chondrosarcomas (Benini et al., 2006; Oshiro et al., 1998), suggesting that tumor suppressor gene inactivation is important in the transformation of enchondromas to malignant sarcomas. p53 regulates cell cycle progression and apoptosis (Komarova et al., 1997), acting as a transcription factor to regulate the expression of genes such as *p21* and *GADD45* and interacting with proteins such as the proapoptotic member of the Bcl2 family, Bax (Kobayashi et al., 2002; Reinke and Lozano, 1997; Srinivasula et al., 1998).

Bone development depends on proper coordination of spatial and temporal control of cell function. The growth plate is an integral component of endochondral bone development and is also responsible for postnatal longitudinal growth. Growth plate chondrocytes undergo an orderly process of proliferation and

SIGNIFICANCE

Although molecular mechanisms responsible for the progression of benign to malignant tumors of epithelial origin have been identified, they have not been demonstrated in mesenchymal tumors. Here, we used a mouse model of enchondromatosis to show that p53 deficiency can cause chondrosarcomas to arise from benign lesions. An unexpected role for IGFBP3 in this progression was found. Human cartilage tumors have low levels of *IGFBP3* expression, compared to normal chondrocytes, with chondrosarcomas having lower levels than benign lesions, suggesting that *IGFBP3* level is a prognostic factor in cartilage tumors. Furthermore, IGFBP3 treatment or IGF signaling blockade increased chondrosarcoma apoptosis, suggesting a therapeutic approach to chondrosarcomas, a tumor for which there is no universally effective chemotherapy.

differentiation, ultimately resulting in the production of extracellular matrix components, such as type X collagen (ColX), and programmed cell death. Bone forms initially in the central part of the bone, adjacent to the metaphyseal growth plate. Secondary ossification sites arise at the epiphyseal ends of the bone and progress toward the center of the bone shaft, eventually leading to closure of the growth plate at maturity. This process is tightly regulated by a number of signaling pathways that maintain the normal rate of endochondral bone growth (Ballock and O'Keefe, 2003).

Because of their location adjacent to the growth plate, it has long been suspected that enchondromas arise from rests of growth plate chondrocytes that fail to undergo terminal differentiation. This notion is supported by the development of metaphyseal cartilage rests, similar in nature to enchondromas in humans, in transgenic mice expressing either a mutant PTHR1 or the hedgehog (Hh)-activated transcription factor Gli2 in chondrocytes (Hopyan et al., 2002). PTHrP and the Hh signaling pathways play important roles in regulating growth plate chondrocyte differentiation. Activation of the PTHrP pathway delays chondrocyte differentiation (Amizuka et al., 1994; Karaplis et al., 1994; Vortkamp et al., 1996), resulting in a downregulation of expression of the Hh ligand, Indian Hedgehog (Ihh), by hypertrophic chondrocytes (Lanske et al., 1996). Ihh regulates chondrocyte proliferation and differentiation though direct regulation of Gli transcription factors and by regulating PTHrP ligand expression (Kobayashi et al., 2002; Koziel et al., 2005; Mau et al., 2007). As such, these signaling pathways act in a feedback loop regulating differentiation. In the case of enchondromatosis, there is deregulation of this feedback loop, resulting in constitutive activation of Hh signaling, even in the presence of PTHrP stimulation and Gli-mediated transcriptional activation (Hopyan et al., 2002; Tiet et al., 2006).

Although malignant and benign cartilage lesions exhibit constitutive Gli-mediated transcription, the level of Gli transcriptional activation is similar for both tumor types, suggesting that while constitutive Hh signaling is a common finding in cartilage tumors, a higher level of activation is not responsible for malignant progression (Tiet et al., 2006). Other factors, such as inactivation of tumor suppressor genes, are thus likely important in cartilage tumor progression. As such, the aim of this study was to investigate the effect of p53 deficiency on the progression of enchondromas.

RESULTS

Deficiency of p53 in Gli2-Transgenic Mice Predisposes to the Development of Larger, More Cellular, Cartilage Lesions

We utilized a mouse model of enchondromatosis in which the Hh-activated transcription factor, *Gli2*, is expressed in chondrocytes driven by the regulatory elements of type two collagen, *Tg(Gli2;Col*II*A)* (Hopyan et al., 2002). The tumors in these mice develop on the basis of a molecular feature that is common in enchondromas—constitutive activation of Gli-mediated transcription—and, as such, they are an applicable model for the human disorder. To determine whether p53 deficiency alters the neoplastic phenotype of cartilage lesions, *Tg(Gli2;Col*II*A)* mice were crossed with mice expressing a null p53 allele (Jacks et al., 1994). The cartilage tumor phenotype was compared between *Tg(Gli2;Col*II*A);p53*$^{+/-}$ mice and littermate *Tg(Gli2;Col*II*A);p53*$^{+/+}$ mice. Since the metaphyseal cartilage lesions occurring in *Tg(Gli2;Col*II*A)* mice are observed at greatest frequency about the distal femur, we examined longitudinal sections of the bone for histological comparison. Three sections, equally distributed across the knee, were analyzed from each limb to allow for a systematic analysis. Cartilaginous lesions were identified in all of the mice. There were an average of 5.8 ± 2.3 lesions in the growth plate of *Tg(Gli2;Col*II*A);p53*$^{+/+}$ mice. These lesions measured under 0.5 mm in diameter (mean diameter, 0.32 ± 0.12 mm) and were composed of cells with occasional binucleate lacunae in a cartilage matrix. There were 12.3 ± 3.8 cells per high-powered field in the lesions (Figures 1A and 1C). Some of the lesions were connected to the growth plate through columns of cartilage. These lesions were identified in mice at all ages observed, up to 12 months of age.

In contrast, *Tg(Gli2;Col*II*A);p53*$^{+/-}$ mice developed larger (greater than 1 mm in diameter) cartilage lesions (mean diameter, 1.7 ± 0.3 mm) (Figures 1B and 1D). They were frequently in contact with the growth plate and had increased cellularity, compared to the smaller lesions (23.4 ± 7.8 cells per high-powered field). These cells showed variability in cytological appearance, seeming to have a broad range of characteristics of chondrocytes present during development (Figure 1F). There were pleiomorphic nuclei in many of the cells. These lesions were not observed in mice at 2 months of age, but were identified in a larger proportion of mice at subsequent time points. At 1 year of age, 50% of mice exhibited these lesions (Figure 1E). The appearance of the larger lesions did not change with the age of the mice (Figure 1E). Overall, this appearance is similar to that of a low-grade chondrosarcoma. We did not find a loss of the normal *p53* allele in these larger lesions, as determined using a PCR-based technique (Figure 1I). The level of *p53* expression was roughly half that of *Tg(Gli2;Col*II*A);p53*$^{+/+}$ littermates, suggesting normal expression from the wild-type allele (Figure 1J).

In a small number of mice (6%), a larger lesion (>1 cm in diameter) arose from bone, extending into the soft tissues. These lesions had a cytological appearance consistent with a higher grade sarcoma, composed of spindle-like cells (Figure 1G). These lesions showed loss of heterozygosity for the wild-type *p53* allele and exhibited a lack of *p53* RNA expression (Figures 1I and 1J).

p53 and Gli2 Interact to Regulate Chondrocyte Proliferation and ColX Expression in the Fetal Growth Plate

To determine whether the change in phenotype was due to alterations in proliferation or differentiation, we examined these parameters in the tumors and distal femurs of fetal limbs. Proliferation was examined in the tumors using phosphohistone H3 staining, showing a trend toward a greater percentage of stained cells in the larger lesions that developed in the *Tg(Gli2;Col*II*A);p53*$^{+/-}$ mice, compared with lesions in *Tg(Gli2;Col*II*A);p53*$^{+/+}$ mice. There was also little difference in the proportion of cells staining for ColX. We examined these same parameters in the growth plate from mature mice and also did not observe substantial differences. Since changes in chondrocyte behavior that could be responsible for the changes in the tumor phenotype may be more readily apparent in fetal limbs, we examined

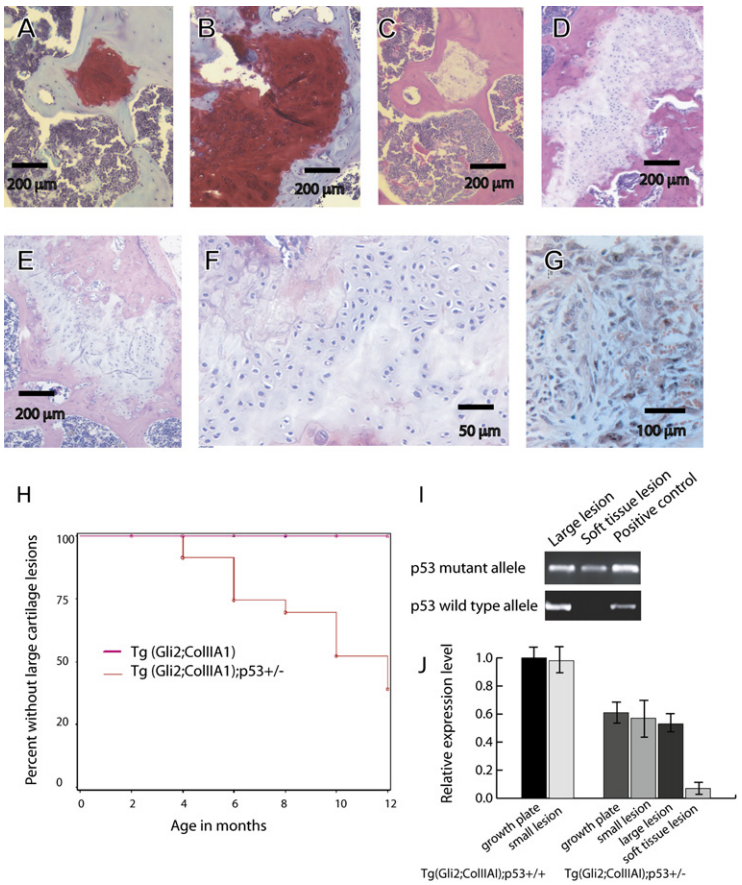

Figure 1. Cartilage Lesions in Mice

(A–G) Histology of tumors in mice overexpressing Gli2 (*Tg(Gli2;ColIIAI);p53*$^{+/+}$) and Gli2-overexpressing mice also deficient in p53 (*Tg(Gli2;ColIIAI);p53*$^{+/-}$). Safranin O staining (A and B) or hematoxylin and eosin staining (C–G) is shown. *Tg(Gli2;ColIIAI);p53*$^{+/+}$ mice demonstrate lesions in the metaphysis that are under 0.5 mm in diameter, are composed of cells with occasional binucleate lacunae in a cartilage matrix, and have an appearance not unlike that seen in human enchondromas (A and C). *Tg(Gli2;ColIIAI);p53*$^{+/-}$ mice developed larger lesions, greater than 1 mm in diameter (B and D). Lesions that were identified in mice at different ages have a similar appearance, as illustrated by a lesion in a 4-month-old (D) and a 12-month-old mouse (E). Higher magnification shows that these larger cartilage lesions have increased cellularity, variability in cytological appearance, pleiomorphic nuclei in many of the cells, and an appearance similar to that of a low-grade chondrosarcoma (F). A small number of mice developed lesions with a soft tissue mass; a histologic appearance consistent with an undifferentiated sarcoma developed in some of the mice, in association with loss of the wild-type p53 allele (G).

(H) Kaplan-Meier survival curve showing the proportion of mice that develop large cartilage lesions at various ages.

(I) The wild-type alleles are lost in the undifferentiated mesenchymal lesions with a soft tissue mass, but not in other cartilage lesions.

(J) Quantitative RT-PCR showing relative level of p53 expression, compared with the growth plate from *Tg(Gli2;ColIIAI);p53*$^{+/+}$ or *Tg(Gli2;ColIIAI);p53*$^{+/-}$ mice. There is a level of expression in *p53*$^{+/-}$ mice that is about half the level in *p53*$^{+/+}$ mice. There is a similar level of expression in the cartilage lesions that formed, compared with the growth plates, regardless of the genotype. There is a substantial decline in the level of expression in the undifferentiated mesenchymal lesions that are associated with a soft tissue mass. Data are given as means and error bars as 95% confidence intervals.

E16.5 limbs. Tg(*Gli2;ColIIAI*) mice had a smaller zone of ColX staining, a finding that was consistent with previous studies of the phenotype of this mouse. Mice deficient in *p53* had a larger zone of ColX expression than did wild-type mice, and, surprisingly, mice overexpressing *Gli2* in the growth plate and deficient in *p53* had an even larger zone of ColX expression (Figures 2A and 2B).

In the fetal limbs, we found that Ki67 staining was a more reproducible marker of proliferation. The relative length of the growth plate, length of the zone expressing ColX, length of the zone staining for Ki67, and proportion of Ki67-stained cells from the distal femur were examined. The length of the proliferative zone (zone with Ki67 staining) did not change significantly with overexpression of *Gli2* or alteration of *p53* protein levels. Within the proliferative zone, there was a trend toward an increase in the percentage of Ki67-stained cells in mice overexpressing *Gli2* or in mice deficient in one or both *p53* alleles (Figure 2C).

Chondrocyte Apoptosis Is Regulated by Both Gli-Mediated Transcription and p53

The decrease in the height of the region of the growth plate expressing ColX in Gli2-overexpressing mice is consistent with the notion that enchondromas are associated with an inhibition in chondrocyte differentiation, maintaining some growth plate chondrocytes in a less-differentiated state. These cartilage islands are left behind as the bone grows, becoming entrapped in the developing metaphysis. The change in ColX staining in the distal femurs from *Gli2*-transgenic, *p53*-deficient mice is, at first glance, at odds with this notion, because an extended region of staining in these mice suggests that cells are entering terminal differentiation at an accelerated rate. However, another possibility is that the larger region of ColX staining is due to a block to cells exiting this zone. Cells could leave this region of the growth through programmed cell death. Inhibition of apoptosis could thus explain the increased zone of ColX staining and could also be responsible for the development of the larger cartilage lesions. To test for this possibility, we examined the expression of markers of apoptosis in the fetal growth plates. The percentage of cells exhibiting either TUNEL or active caspase-3 staining was moderately decreased in the growth plate of both Gli2-overexpressing and p53$^{-/-}$ mice. However, both TUNEL staining and active caspase-3 staining were substantially decreased in *Tg(Gli2;ColIIAI);p53*$^{-/-}$ mice (Figures 3A, 3B, and 3C). Thus, p53 and Gli2 cooperate to regulate apoptosis in chondrocytes, with Gli2 being a negative regulator and p53 a positive regulator of this process. The length of the ossified portion of the fetal femurs was compared with the overall bone length. There was a decrease in the proportion of ossified bone in the limbs showing a decrease in apoptosis, supporting the notion that decreased apoptosis is the cause of the increase in length of ColX staining (Figure 3D).

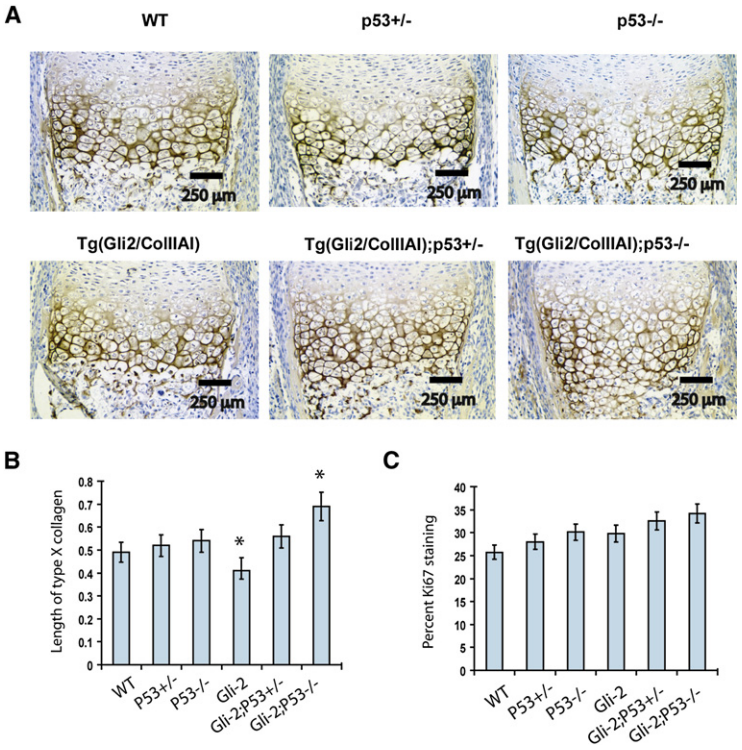

Figure 2. p53 and Gli2 Regulate Growth Plate Proliferation and ColX Expression in the Growth Plate

(A and B) The length of the region of the growth plate exhibiting ColX staining, a measure of differentiation. Representative histological sections are shown (A). The height of the region exhibiting ColX staining in *Tg(Gli2;ColIIAI)* mice was significantly smaller than that in wild-type (WT) mice, whereas $p53^{-/-}$ mice had a longer zone than did WT mice, although this did not reach statistical significance. *Tg(Gli2;ColIIAI)*;$p53^{-/-}$ mice had an significantly longer zone of ColX expression than did WT mice. Graphical representation of the heights of the ColX staining in the growth plates, with data given as means and 95% confidence intervals, is also shown (B). Asterisks are above data that are significantly different from WT mice.

(C) Proliferation as measured by average percentage of Ki67-positive cells in the various genotypes and error bars as 95% confidence intervals, although these did not reach statistical significance.

IGFBP3 Is a Downregulated Gene in Cartilaginous Neoplasia

Gene profiling was used to identify genes differentially regulated under PTHrP stimulation by the R150C variant PTHR1, a mutant PTHrP receptor found in rare cases of enchondromatosis (Hopyan et al., 2002; Couvineau et al., 2008). Gene expression was compared between C3H10T1/2 cells stably transfected with either the R150C variant PTHR1 or a wild-type PTHR1, using a technique described elsewhere (Mau et al., 2007). A small number of genes were differentially regulated by at least 3-fold. One of the genes identified, a known p53 target, was *Igfbp3*. The differential expression of this gene was confirmed in the cell lines using quantitative RT-PCR.

To determine whether *IGFBP3* was differentially regulated in human cartilage tumors, we examined 6 enchondromas and 29 chondrosarcomas of various grades and compared the level of expression with normal growth plate cartilage from 4 patients.

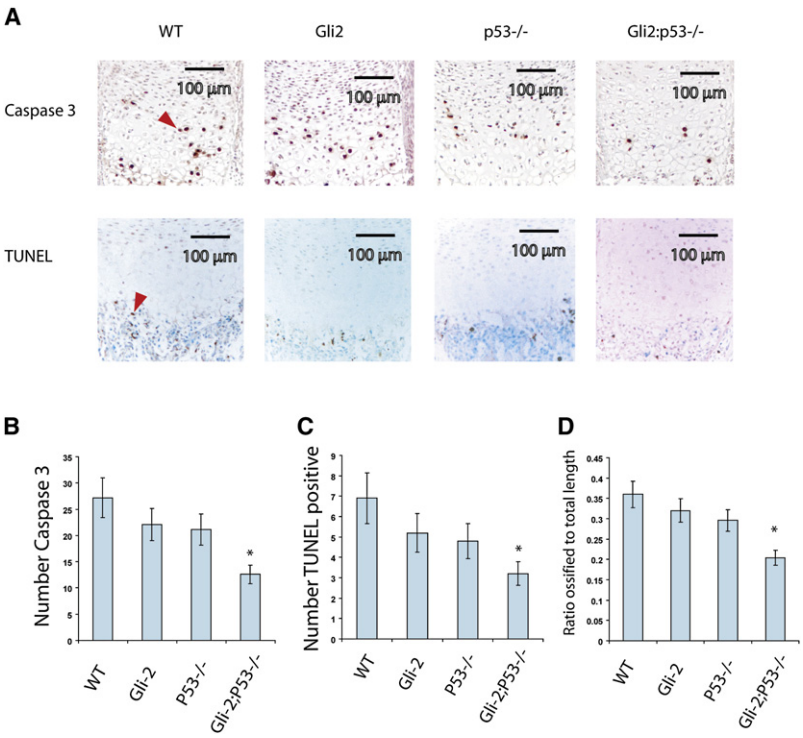

Figure 3. p53 and Gli2 Regulate Growth Plate Apoptosis

(A) Representative histologic sections. The arrows show positively stained cells.

(B and C) Number of TUNEL-positive and caspase-3-positive cells was compared between *Tg(Gli2;ColIIAI)*;$p53^{-/-}$, *Tg(Gli2;ColIIAI)*;$p53^{+/+}$, and $p53^{-/-}$ littermate mice. There was a decrease in both TUNEL and active caspase-3 staining in *Tg(Gli2;ColIIAI)* and $p53^{-/-}$ mice, compared with WT mice, and there was a further increase in staining in *Tg(Gli2;ColIIAI)*;$p53^{-/-}$ mice. Data are given as means and 95% confidence intervals. Asterisks are above data that are significantly different from both *Tg(Gli2;ColIIAI* or $p53^{-/-}$ mice.

(D) The ratio of the ossified to total length of the bone. Asterisks are above data that are significantly different from both *Tg(Gli2;ColIIAI* or $p53^{-/-}$ mice.

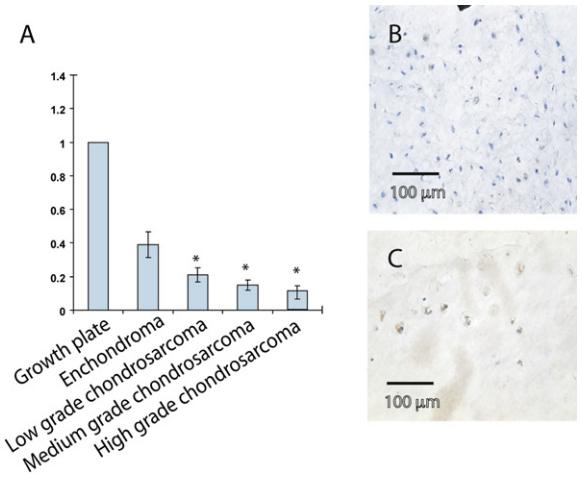

Figure 4. IGFBP3 Is Expressed at Low Levels in Cartilage Tumors

(A) Using quantitative PCR, there was a lower level of expression of *IGFBP3* in cartilage lesions, compared with that in normal growth plates. The level of expression was lower in benign enchondromas than in normal growth plate cartilage, but was even lower in chondrosarcoma. Data are given as means and 95% confidence intervals. An asterisk above a data point shows a significant difference from the level of expression in enchondromas.

(B and C) IGFBP3 immunohistochemistry showed a lower level of expression in a low-grade chondrosarcoma (B) than in a growth plate chondrocyte (C).

There was a decrease in the level of expression in enchondromas and a further decrease in the level of expression in chondrosarcomas (Figure 4A). Importantly, there was a lower level of expression in high-grade lesions than in lower-grade lesions. Immunohistochemistry using an anti-IGFBP3 antibody confirmed the decreased protein level in patient samples (Figure 4B).

IGFPB-3 Expression Is Regulated by Gli-Mediated Signaling

Although previous studies showed that IGFBP3 expression is regulated by p53, it is not known whether Gli-mediated transcription could also regulate its expression. The Mulan website (http://mulan.dcode.org) was used to examine the promoter region of IGFBP3 (Villafuerte et al., 1997). A Gli consensus sequence-binding region that was conserved between humans and mice was identified 1.2 kb upstream of the start site (Figure 5A). Chromatin immunoprecipitation (ChIP) with primer pairs adjacent to this potential binding site and an antibody to Gli2 showed that Gli2 could bind to this site (Figure 5B). To determine whether Gli2 and p53 would regulate expression of *Igfbp3* in vivo, its expression in the cartilage from E16.5 distal femurs of Tg*(Gli2;ColIIAI);p53*$^{-/-}$, *Tg(Gli2;ColIIAI);p53*$^{+/+}$, *p53*$^{-/-}$, and wild-type littermate mice was examined. Both p53 and Gli independently regulated *Igfpb3,* with p53 acting as a positive regulator and Gli acting as a negative regulator of expression (Figure 5C). Explant cultures of chondrosarcoma tumors were established as reported elsewhere (Tiet et al., 2006), and it was found that stimulation with Hh ligand downregulated expression of *IGFBP3*, whereas treatment with an Hh neutralizing antibody, or cyclopamine, which blocks Hh signaling downstream of its receptor, upregulated its expression, compared with controls (Figure 5D). To determine whether hedgehog signaling would directly regulate IGFBP3 expression, a 1.8 kb promoter construct driving luciferase (Cohick et al., 2000) was transfected into NIH 3T3 cells, which were treated with cyclopamine or hedgehog ligand. Hh blockade activated the reporter construct, and Hh ligand decreased its activity. IGF stimulation was used as a positive control (Figure 5E). Using site-directed mutagenesis, this binding site was obliterated, and Hh modulation no longer regulated the reported construct activation, whereas IGF stimulation still caused activation.

IGFBP3 Regulates Apoptosis in the Growth Plate and Neoplastic Chondrocytes

IGFBP3 is known to promote apoptosis and inhibit cell growth in various tissue types (Bhattacharyya et al., 2006; Lee et al., 2005). Studies in chondrocyte cell cultures show a complex relationship between IGFBP3 and IGF in the regulation of proliferation; however, its effect on apoptosis has not been investigated (Kiepe et al., 2002). To examine its function in the growth plate and in cartilaginous neoplasia, we treated wild-type and transgenic limb explants with recombinant IGFBP3. Wild-type mice did not show a difference in proliferation, apoptosis, or size of the zone expressing ColX following IGFBP3 treatment. In contrast, although there was not a significant difference in cell proliferation in *Gli2*-transgenic, *p53*-deficient mice with IGFBP3 treatment, there was a substantial increase in the rate of apoptosis as measured by the number of cells exhibiting TUNEL or active caspase-3 staining. There was also a smaller zone of ColX staining. Indeed, IGFBP3 treatment increased the apoptosis rate to a level similar to that found in wild-type mice (Figure 6). To determine whether IGFBP3 could function in a similar manner in neoplastic chondrocytes, we treated chondrosarcoma cells with IGFBP3 and found that this treatment substantially increased tumor cell apoptosis, as determined using TUNEL staining and caspase-3 activity (Figures 7A, 7B, and 7C). To better determine whether IGFBP3 mediates the effects of p53 and Hh signaling on apoptosis, primary chondrosarcoma cells were transfected with either a p53 expression vector, an siRNA construct targeted to IGFBP3, or control vectors, and then were treated with either the Hh-neutralizing antibody, IGFBP3, or controls. Apoptosis was determined using annexin V staining, as detected using flow cytometry. There was a substantial increase in the proportion of cells exhibiting annexin V staining when expressing p53 and treated with an Hh-neutralizing antibody. In cells expressing siRNA for IGFBP3, there was no change in apoptosis, suggesting that IGFBP3 mediates the effect of Hh and p53 on apoptosis. Treatment of the primary cell culture with IGFBP3 alone increased the apoptosis rate to about the same level as in cells expressing p53 and treated with cyclopamine (Figure 7D). Taken together, these results show that IGFBP3 mediates the effect of p53 and Hh signaling in chondrocytes.

IGFBP3 Regulates Apoptosis through an IGF-Mediated Mechanism

One mechanism by which IGFBP3 can regulate apoptosis is through its interaction with IGF, resulting in the inhibition of IGF signaling through a receptor-mediated mechanism (Kim et al., 2004; Rajah et al., 1997; Silha et al., 2006). To determine whether IGFBP3 mediates IGF signaling, we examined the regulation of insulin receptor substrate-1 (IRS-1) phosphorylation at Y612

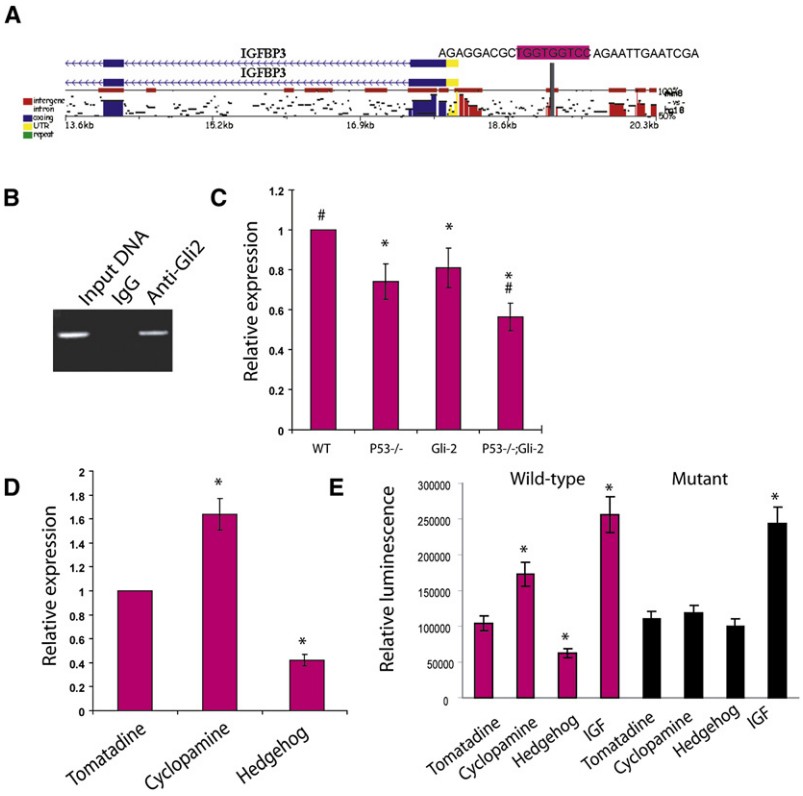

Figure 5. Gli Transcriptional Activation Regulates IGFBP3 Expression

(A) Diagrammatic representation of the IGFBP3 gene, including the promoter region, showing a Gli consensus binding site 1.2 kb downstream of the start site (from MULAN analysis). The binding site is in a DNA region conserved between multiple species.

(B) ChIP shows amplification of the Gli-binding site in the IGFBP3 promoter with use of the Gli-2 antibody but not the control DNA. Additional controls included a second set of primers that crossed the Gli-binding site (positive) and a second set of primers amplifying a randomly selected DNA sequence on the same chromosome (negative).

(C) *Igfbp3* expression in fetal limbs from various mice as determined using quantitative PCR. Data are given as means and 95% confidence intervals. There was a significantly different level of expression between WT mice and each of the genotypes (as indicated with an asterisk above the data) and a significant difference between either the $P53^{-/-}$;GLI-2 mice or WT mice, compared with either $P53^{-/-}$- or Gli-2-transgenic mice (indicated by a number sign above the data point), showing an additive effect of Gli2 and $P53^{-/-}$ on *Igfbp3* expression level.

(D) *IGFBP3* expression level in a chondrosarcoma cell culture treated with Hh blockade using cyclopamine, Hh ligand, tomatadine as a control. Data are given as means and 95% confidence intervals, an asterisk above the data shows significant difference from tomatadine.

(E) Relative luciferase activity produced by an IGFBP3 promoter reporter constructs. Data are given as means and 95% confidence intervals. There is a significant difference in activity between Hh blockage and stimulation, compared with control conditions in the WT construct, but no change in the mutant construct lacking the Gli consensus binding site. An asterisk above the data shows significant difference from tomadaine. IGF treatment is a positive control.

(Yamanaka et al., 1997). Protein isolated from cartilage of fetal limbs showed that phospho-Y612 IRS level is increased in Gli2-transgenic and $p53^{-/-}$ mice, compared with wild-type mice (2.5 and 2.8 times, respectively; $p = 0.02$). The phosphorylation level is further elevated in $p53^{-/-}$ mice that also express Gli2 (3.9 times control; $p = 0.01$) (Figure 8A). To determine whether IGFBP3 mediates IRS-1 phosphorylation in chondrosarcomas, explant cultures were treated with IGFBP3, and this treatment inhibited IRS-1 phosphorylation (Figure 8B). This finding raises the possibility that IGFBP3 is acting through IGF ligand blockade to regulate apoptosis. Limb explants were treated with either IGFBP3 or neutralizing antibodies to IGF-1 and IGF-2. Treatment with neutralizing antibodies caused a similar change in apoptosis, as measured using TUNEL or caspase-3 staining (Figures 8C and 8D). To determine whether IGF blockade would have a similar effect in chondrosarcomas, explants were treated with either IGFBP3 or IGF neutralizing antibodies. There was a similar increase in apoptosis rate with IGF blockade as with IGFBP3 treatment (Figures 8E and 8F). To determine whether IGF ligands would have an effect on cartilage tumor formation, the *Tg(Gli2;ColIIAI)* mouse was crossed with an $Igf2^{+/-}$ mouse (DeChiara et al., 1990) to generate *Tg(Gli2;ColIIAI)*;$Igf2^{+/-}$ mice and *Tg(Gli2;ColIIAI)*;$Igf2^{+/+}$ littermates. There was a substantial decline in the number of cartilage lesions that developed in the mice deficient in IGF2 (Figure 8 G). In addition, the apoptosis rate in growth plate chondrocytes was higher in mice heterozygous for Igf2 (Figures 8H and 8I). Taken together, these data suggest that the effect of IGFBP3 on its regulation of chondrocyte apoptosis is related to its ability to inhibit IGF signaling.

DISCUSSION

The cartilage lesions in mice that overexpress Gli2 in chondrocytes likely occur because some growth plate cells fail to undergo apoptosis and remain behind in the developing bone. The absence of progression of these lesions in mice, in concert with data from human tumors showing a similar level of expression of Hh-regulated genes in both benign and malignant lesions (Tiet et al., 2006), suggests that constitutive Hh signaling activation in chondrocytes on its own is not sufficient to cause a malignant cartilage tumor. Here, we demonstrate that the loss of one p53 allele in Gli2-transgenic mice causes the development of lesions similar to low-grade chondrosarcomas. Furthermore, higher-grade sarcomas developed in association with somatic loss of both p53 alleles. Thus, additional genetic events are necessary for the progression of benign cartilage tumors.

Growth plate chondrocytes differentiate in a tightly regulated manner. While it is thought that apoptosis is responsible for the disappearance of chondrocytes at the end of terminal differentiation, alternative explanations, such as transdifferentiation of chondrocytes to other cell types, have been proposed (Erenpreisa and Roach, 1996; Kahn and Simmons, 1977). Enchondromas that develop in mice are associated with an inhibition of

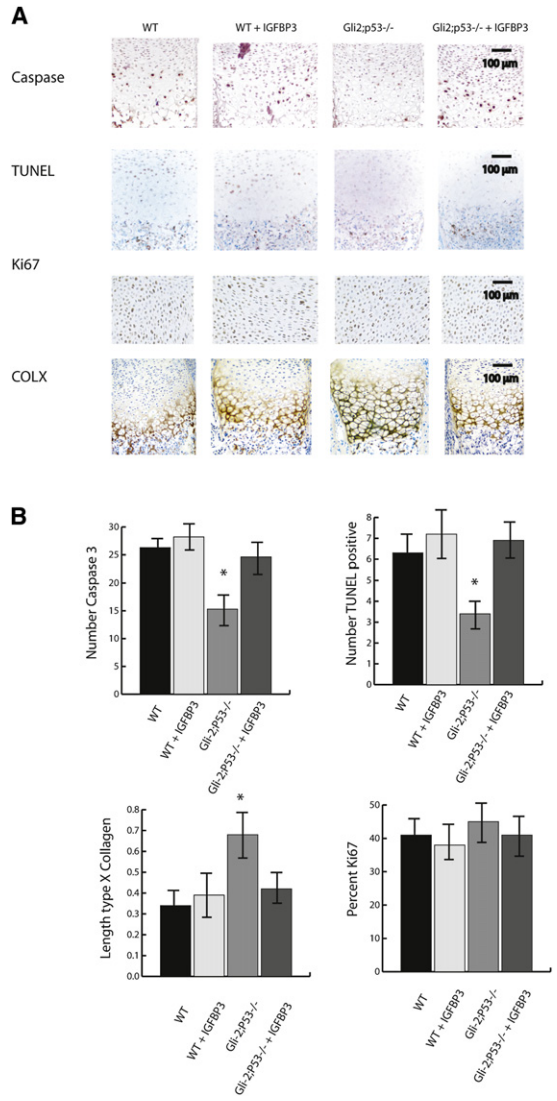

Figure 6. IGFBP3 Regulates Cell Apoptosis and ColX

(A) Representative histologic sections of explant cultures from fetal limb explants shows that IGFBP3 treatment upregulates cell apoptosis and regulates the length of ColX expression in fetal limbs. A positive apoptotic cell is labeled with an arrow.

(B) The rate of apoptosis, Ki67 staining, and length of ColX staining in *Tg(Gli2;ColIIAI);p53*$^{-/-}$ limbs. Data are given as means and 95% confidence intervals. Asterisks are above data that are significantly different from WT mice. Apoptosis and ColX staining in *Tg(Gli2;ColIIAI);p53*$^{-/-}$ limbs returns toward WT levels with IGFBP3 treatment. Treatment of WT limbs had a minimal impact, suggesting a threshold effect of IGFBP3.

growth plate chondrocyte terminal differentiation, which is manifested by a smaller zone of ColX expression in fetal limbs (Hopyan et al., 2002). We found that mice overexpressing Gli2 that are also deficient in p53 develop enchondromas as well as lesions similar in histological appearance to low-grade chondrosarcomas. Surprisingly, the fetal growth plate in these mice shows an increased length of ColX expression. One explanation for this phenotype is that cells are delayed from leaving the zone of ColX by an inhibition of apoptosis. Our data showing an inverse relationship between apoptosis rate and length of ColX staining in these mice suggest that apoptosis is indeed responsible for chondrocytes exiting the zone of terminal differentiation.

One possible mechanism by which Gli and p53 could act to modulate changes in apoptosis is through the combined regulation of expression of a common target gene. We identified *IGFBP3* as a gene that is differentially regulated by both Gli-mediated signaling and p53. While IGFBP3 is known to be expressed in the growth plate (Olney and Mougey, 1999; Parker et al., 2007), its function in growth plate regulation had not previously been elucidated. Fetal limb explant experiments showed that IGFBP3 regulates chondrocyte apoptosis. It is possible that in the normal growth plate, IGFBP3 expression acts to sequester IGF near the zone of terminal differentiation, allowing normal apoptotic cell death. Such a function may explain previous cell culture data showing a lack of effect of IGFBP3 in regulating cell proliferation after IGF stimulation in terminally differentiated chondrocytes, whereas it has such an effect in proliferating chondrocytes (Kiepe et al., 2002; Spagnoli et al., 2001), as the overriding function of IGFBP3 in terminally differentiated cells may be to allow for cell death.

IGFBP3 can promote apoptosis and inhibit cell growth in various tissue types (Jenkins et al., 2005; Kiepe et al., 2002; Lee et al., 2002; MacLean et al., 2003; Shim et al., 2004; Silha et al., 2006; Spagnoli et al., 2001) through IGF-dependent or -independent mechanisms. As a carrier of free IGFs, IGFBP3 may inhibit cell survival by sequestering IGFs from activating IGF receptors. Given that IGFBP3 regulates IRS phosphorylation and induces similar apoptotic changes in chondrocytes to that of IGF-neutralizing antibody treatment, it is possible that, in these cell types, IGFBP3 acts through IGF to regulate aptotosis. Nevertheless, there are other potential mechanisms by which Hh signaling can regulate apoptosis. In vitro studies have suggested a direct modulation of transcriptional activity of proapoptotic Bcl-2 (Bar et al., 2007; Bigelow et al., 2004; Kasper et al., 2006; Louro et al., 2002) and, more recently, the posttranscriptional stabilization of Bcl-2 and Bcl-X_L that is dependent on PI3-kinase signaling (Morton et al., 2007).

Hh signaling could regulate IGF activity through other mechanisms. For instance, Hh signaling in medulloblastoma precursor cells acts through mTOR to regulate IGFR-1 signaling (Parathath et al., 2008), and in prostate development, Hh signaling regulates IGFBP-6 expression (Lipinski et al., 2005). Taken in conjunction with our findings, this suggests that multiple mechanisms of cross-regulation exist between Hh and IGF signaling. Gli-mediated transcription acts through *IGFBP3* to regulate apoptosis and may also play a role in other developmental and neoplastic processes. For instance, the finding that, in prostate cancer cell lines, Hh blockade results in inhibition of IGF activity (Levitt et al., 2007) might be explained by this mechanism.

In support of an important role for IGFBP3 and IGF signaling in sarcomas, in vitro studies demonstrated growth inhibitory and proapoptotic functions in Ewing's sarcoma (Benini et al., 2006) and osteosarcoma (Schmid et al., 2001) cell lines. In addition to its effect in sarcoma cell lines, the use of recombinant IGFBP3 in other tumor types and in animal models of neoplasia resulting in an anticancer effect is reported. (Butt et al., 2000; Jerome et al., 2003, 2006; Rajah et al., 1997; Vokes et al., 2007). We found that IGFBP3 primarily regulates apoptosis in chondrosarcomas through inhibition of IGF signaling. Since inhibition of Hh

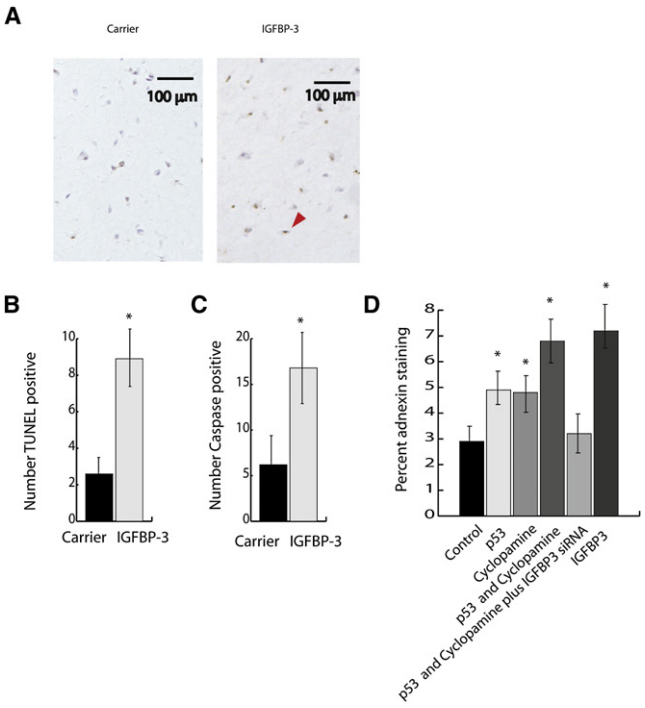

Figure 7. IGFBP3 Regulates Chondrosarcoma Apoptosis

(A) Representative histologic sections showing TUNEL staining from organ cultures of chondrosarcomas treated with IGFBP3 or carrier, showing a substantial increase in apoptosis rate with IGFBP3. Arrow shows a TUNEL-positive cell.

(B and C) Mean and 95% confidence intervals for the number of TUNEL-positive cells or active caspase-3-positive cells per high-powered field. There is a statistically significant difference in TUNEL staining and caspase staining with IGFBP3 treatment. An asterisk is above data that are significantly different from the control.

(D) Mean and 95% confidence intervals for proportion of cells exhibiting annexin V staining, as detected using flow cytometry in primary cell cultures from a chondrosarcoma. siRNA for IGFBP3 brings the percentage of staining in cells expressing p53 and treated with a Hh neutralizing antibody to baseline, suggesting that the regulation of apoptosis by p53 and Hh signaling is mediated by IGFBP3. An asterisk is above data that are significantly different from control data.

signaling reduces chondrosarcoma cell proliferation, this raises the intriguing possibility that combined treatment with Hh blockade may produce synergistic antitumor effects. Such a treatment would be particularly important in chondrosarcoma, as there are no universally effective chemotherapies for this tumor type (Wunder et al., 2007). Furthermore, we found that *IGFBP3* expression is dramatically reduced in human cartilage tumors and that its level of expression is lower in chondrosarcomas than in benign enchondromas. This raises the possibility that the level of expression of IGFBP3 can be used as a prognostic factor and a useful adjunct in the pathologic evaluation of cartilage lesions for distinguishing between tumor grades.

EXPERIMENTAL PROCEDURES

Human Samples

Cartilage tumor samples and normal human growth plate cartilage were prepared for organ culture or were cryopreserved immediately after surgical excision. The cryopreserved samples were used in a previous study of Hh signaling, and additional clinical information has been reported elsewhere (Tiet et al., 2006). Six chondrosarcoma samples were analyzed for explant experiments, and each was analyzed in triplicate. An additional three chondrosarcoma samples were prepared as a primary cell culture, as reported elsewhere (Wu et al., 2007). Informed consent was obtained from all patients prior to surgery. These studies were approved by the Mount Sinai Hospital Research Ethics Review Board and The Ethics Review Board Research Institute Hospital for Sick Children.

Mice

A mouse model of enchondromatosis in which the Hh-activated growth factor, Gli2, is expressed in growth plate chondrocytes driven by the regulatory elements of type two collagen, Tg(Gli2;ColIIAI), was used. Tumors develop in these mice according to a molecular mechanism that is a common feature in enchondromas; as such, these tumors are an applicable model for the human disorder (Hopyan et al., 2002). *rp53^{tm1Tyj}* mice (*p53$^{+/-}$*) mice (Jacks et al., 1994) were crossed with Tg(Gli2;ColIIAI) mice, and progeny were bred to generate *Tg(Gli2;ColIIA1);p53$^{-/-}$*, *Tg(Gli2;ColIIA1);p53$^{+/-}$*, *Tg(Gli2;ColIIA1);p53$^{+/+}$*, *p53$^{+/-}$*, *p53$^{-/-}$*, and wild-type littermates using a previously reported crossing strategy (Poon et al., 2001). The tumor phenotype between at least eight littermates of each genotype and age group were compared. Very few *Tg(Gli2;ColIIA1);p53$^{-/-}$* or *p53$^{-/-}$* mice were available for analysis, and thus they were excluded from our tumor phenotypic analysis. For analysis of fetal limbs, mice at E16.5 were examined, and at least six mice of each genotype or treatment condition were analyzed. *Igf2^{tm1Rob} (Igf$^{+/-}$)* mice (DeChiara et al., 1990) were crossed with *Tg(Gli2;ColIIA1)* mice in a similar manner to generate *Tg(Gli2;ColIIA1);Igf2$^{+/-}$* and *Tg(Gli2;ColIIA1);Igf2$^{+/+}$* mice. A mouse protocol describing the above experimental procedures was approved by the animal care committee of The Hospital for Sick Children.

Explant Analysis

Hind limbs from E16.5 embryos were placed in DMEM containing 0.1% BSA, vitamin C (50 mg/ml), and Antibiotic-Antimycotic Solution (Wisent). One limb from each embryo was treated with Hh ligand, IGFBP3, or IGF-1, whereas the other acted as a control. Shh and anti-Hh antibody (monoclonal antibody, Hybridoma Bank, University of Iowa, Iowa City, IA), cyclopamine, or tomatadine (a cyclopamine analog that lacks the ability to block Hh signaling as a control) were used as reported elsewhere (Mau et al., 2007; Tiet et al., 2006). Shh and the Hh antibody will cross-react to activate or inactivate Ihh signaling (Tiet et al., 2006). Human or mouse recombinant IGFBP3 (2 μg/ml), IGF-I neutralizing antibody (10 μg/ml), or IGF-II neutralizing antibody (10 μg/ml) were obtained from R&D systems and utilized as reported elsewhere (Karey and Sirbasku, 1988). The agents were used alone or in combination for three consecutive days, with media changed each day replenishing the specific agent utilized.

Histological Analysis, Immunohistochemistry, and Western Analysis

Samples were fixed in 4% paraformaldehyde, treated with 20% EDTA for decalcification, embedded in paraffin, and sectioned for histological evaluation. Safranin O and hematoxylin and eosin staining was performed using standard techniques. ColX staining was used as a marker for chondrocyte terminal differentiation, by incubating the sections with a 1:50 dilution of anti-human recombinant ColX (Quartett Immunodiagnostika Biotechnologie GMBH) at 4°C overnight. Proliferation in fetal limbs was assayed using a Ki-67 antibody monoclonal antibody at a 1:50 dilution (DaykoCytochemical, M7249, Clone TEC-3) at 4°C overnight. Deparaffinized sections of decalcified mature limbs were incubated with a 1:200 dilution of a phospho-histone H3 antibody (Sigma Chemical Company, St. Louis) at 4°C overnight and were counterstained with hematoxylin. IGFBP3 protein was detected using a rabbit anti-mouse antibody (Cell Sciences, Canton, MA, Cat #PAK-1) and incubated at a 1:300 dilution at 4°C overnight. Western analysis for IRS was undertaken using an anti-phospho Y612 IRS-1 antibody at a 1:1000 dilution (Abcam).

Caspase-3, TUNEL, and Annexin V Assays

Active Caspase-3 and TUNEL staining was used to detect apoptotic cells in tissue sections. Deparaffinized sections were stained using an anti-active caspase-3 antibody (Promega, Madison, WI, Cat #G7481) or using the Apoptag

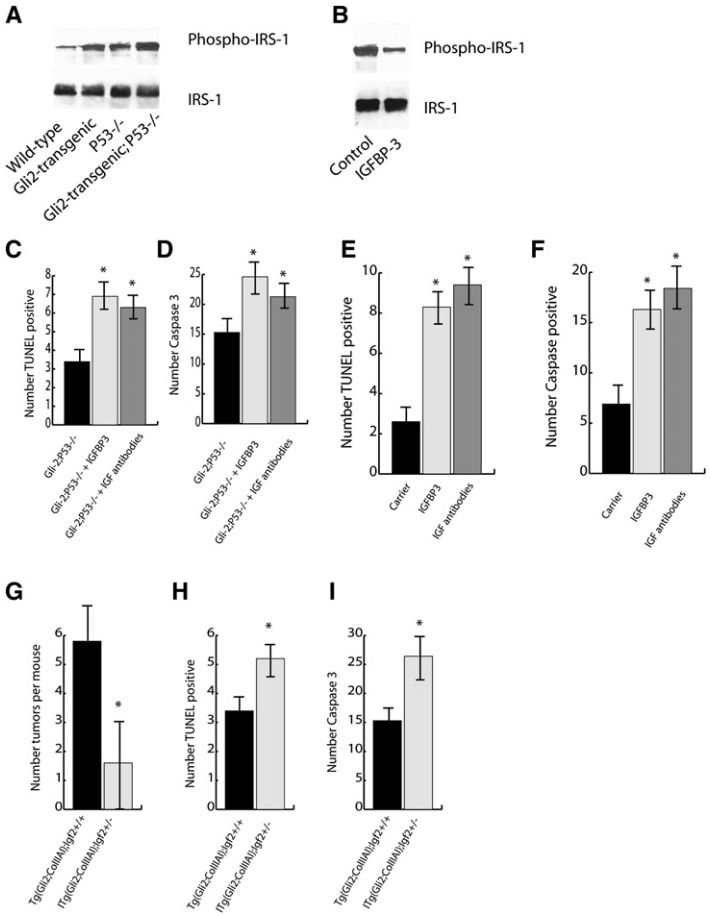

Figure 8. IGF Signaling Regulates Chondrocyte Apoptosis and Cartilage Tumor Formation

(A) Western analysis for phosphorylated and total insulin receptor substrate-1 (IRS-1) from protein extracts from various mouse genotypes showing that IRS-1 phosphorylation level is increased in *Tg(Gli2;ColIIAl)*, $p53^{-/-}$ and *Tg(Gli2;ColIIAl)*;$p53^{-/-}$ mice.

(B) Western analysis for phosphorylated IRS-1 in protein extracts from chondrosarcoma explants treated with IGFBP3 or carrier showing a lower phosphorylation level with IGFBP3 treatment.

(C and D) Mean and 95% confidence intervals for the number of TUNEL-positive cells or caspase-positive cells in growth plates from explant limbs of treated with IGFBP3 or neutralizing antibodies IGF-1 and IGF-2, showing a similar change in apoptosis with use of the neutralizing antibodies as with IGFBP3. An asterisk above a data point shows a significant difference from control conditions.

(E and F) Mean and 95% confidence intervals for the number of TUNEL-positive cells or caspase-positive cells per high-powered field in chondrosarcoma explants treated with either IGFBP3 or IGF neutralizing antibodies. An asterisk above a data point shows a significant difference from control conditions.

(G) The mean number and 95% confidence intervals of cartilage lesions in the femur of *Tg(Gli2;ColIIAl)*;$Igf2^{+/-}$ mice and *Tg(Gli2;ColIIAl)*;$Igf2^{+/+}$ littermates. There was a substantial decline in the number of cartilage lesions that developed in the mice deficient in IGF2.

(H and I) Mean and 95% confidence intervals for the number of TUNEL- or caspase-stained cells in growth plates from *Tg(Gli2;ColIIAl)*;$Igf2^{+/-}$ mice and *Tg(Gli2;ColIIAl)*;$Igf2^{+/+}$ littermates. An asterisk above a data point in (G)–(I) indicates a significant difference from Tg(Gli2;ColIIAl);Igf2$^{+/+}$ mice.

Plus Peroxidase In Situ Apoptosis Detection Kit (Chemicon International), according to the manufacturer's instructions. The proportion of cells exhibiting annexin V staining was determined in cell cultures, using flow cytometry as previously reported (Tjandra et al., 2007).

Cell Transfection and Reporter Constructs

A 1.8 kb IGFBP3 promoter construct driving luciferase (Cohick et al., 2000) was transfected using lipofection. The QuikChange site-directed mutagenesis kit (Stratagene) was used to mutate the putative Gli-binding site in this construct as a negative control. Cells were then treated with cyclopamine, Shh ligand, Hh antibody, or tomatadine (as a control) for 24 hr, and luciferase levels were determined. A p53 expression vector was obtained from Clontech (Mountain View, CA) and was transfected into primary chondrosarcomas (Itoh et al., 2004). siRNA constructs for IGFBP3 (Santa Cruz, CA) were used as per the manufacturer's recommendations. A GFP-expressing construct was used to identify successfully transfected cells for flow cytometry analysis.

Microarray Analysis

For gene profiling studies, the cell line was stably transfected with either the R150C variant PTHR1 or a wild-type PTHR1, as previously reported (Mau et al., 2007). RNA isolated from the samples was converted to double-stranded cDNA using Superscript (GIBCO-Invitrogen) with a T7-$(dT)_{24}$ primer, which was then transcribed to biotinylated complementary RNA (cRNA) by incorporating biotin-CTP and biotin-UTP using Enzo BioArray High Yield RNA labeling kit (Enzo Diagnostics, New York). The cRNA labeling and hybridizations were then performed according to Affymetrix GeneChip Protocol (Affymetrix Inc., Santa Clara, CA). The chips were scanned for fluorescence signal detection. The experiment was performed in triplicate, and a 3-fold expression difference was arbitrarily used as a threshold to detect differentially regulated genes.

Real-Time Quantitative PCR

RNA was isolated from at least three independent experiments and was analyzed in separate PCRs, with a minimum of triplicates for each treatment condition and primer set. Gene expression between samples was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Either the 28S rRNA or β-actin gene was used for target gene normalization.

ChIP Analysis

ChIP assay was performed using the ChIP-IT kit (Active Motif) according to the manufacturer's protocol. Cells were fixed with 1% formaldehyde to preserve protein/DNA interactions. The protein/DNA complex was then sheared with eight pulses at power level two using a Sonic Dismembrator (Fisher Scientific) of 10 s sonication followed by a 30 s rest on ice. A Gli2 rabbit polyclonal antibody (Abcam) and negative control IgG (Active Motif) were used to immunoprecipitate the DNA/protein complex. Following immunoprecipitation, crosslinking was reversed, the proteins were removed by treatment with Proteinase K, and the DNA was purified and analyzed using PCR.

p53 Loss of Heterozygosity by PCR

Tumors in Tg(Gli2;ColIIAl);$p53^{+/-}$ mice were analyzed for loss of a p53 allele using the same primers used for genotyping mice. DNA was extracted from the tumor using GenElute Mammalian Genomic DNA Miniprep kit (Sigma, Chemical Company, St. Louis) and subsequently analyzed by PCR.

Statistical Analysis

Means and 95% confidence intervals were calculated. The plus-or-minus sign (±) is used in the text to show the 95% confidence intervals. The student t test was used for comparison of data sets. A p value of <0.05 was used as a threshold for statistical significance.

ACCESSION NUMBERS

Array data are deposited in the GEO database, accession number GSE15118 (http://www.ncbi.nlm.nih.gov/geo/).

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