Proteoglycan desulfation determines the efficiency of chondrocyte autophagy and the extent of FGF signaling during endochondral ossification

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Cartilage extracellular matrix (ECM) contains large amounts of proteoglycans made of a protein core decorated by highly sulfated sugar chains, the glycosaminoglycans (GAGs). GAGs desulfation, a necessary step for their degradation, is exerted by sulfatases that are activated by another enzyme, Sulfatase-Modifying Factor 1 (SUMF1), whose inactivation in humans leads to severe skeletal abnormalities. We show here that despite being expressed in both osteoblasts and chondrocytes *Sumf1* does not affect osteoblast differentiation. Conversely, in chondrocytes it favors ECM production and autophagy and promotes proliferation and differentiation by limiting FGF signaling. Thus, proteoglycan desulfation is a critical regulator of chondrogenesis.

Endochondral ossification, a multistep process responsible for embryonic bone formation and postnatal longitudinal bone growth (Kronenberg 2003), begins once mesenchymal cells condense, forming the blueprint of the future skeleton. Then, these mesenchymal cells differentiate into resting and proliferating chondrocytes that express *Aggrecan* and $\alpha_1(II)$ *Collagen* while cells at the periphery express $\alpha_1(I)$ *Collagen* and form the perichondrium. Subsequently, chondrocytes in the center of this structure further differentiate into hypertrophic chondrocytes expressing $\alpha_1(X)$ *Collagen* but not *Aggrecan* and $\alpha_1(II)$ *Collagen*. The extracellular matrix (ECM)

secreted by hypertrophic chondrocytes allows vascular invasion, degradation of the calcified ECM, and initiation of osteogenesis. Thus, the ECM plays important functions during skeletal development (Olsen et al. 2000).

Alongside collagen fibrils the cartilagineous ECM contains large amounts of proteoglycans that protect these fibrils and provide resistance against compression. Proteoglycans are composed of a protein core to which one or more highly sulfated polysaccharide chains (glycosaminoglycan [GAG]) bind. The GAGs moiety of proteoglycans can regulate the distribution and binding ability of several signaling molecules, thereby influencing developmental processes. GAGs fulfill these functions according to their degree of sulfation (Esko and Selleck 2002; Perrimon and Hacker 2004) a process determined by two classes of intracellular enzymes: the sulfotransferases, that catalyze incorporation of sulfated groups into a nascent molecule, and the sulfatases, that remove them (Diez-Roux and Ballabio 2005; Bulow and Hobert 2006).

There are at least 17 different sulfatases in vertebrates, whose function is determined by their subcellular localization in the lysosomes, cell surface, or ER/Golgi, and by substrates specificity (Diez-Roux and Ballabio 2005). In addition, sulfatase becomes active following a posttranslational formylglycination catalyzed by the enzyme Sulfatase-Modifying Factor 1 (Sumf1) (Cosma et al. 2003). To date, no other substrates besides the sulfatases have been identified for Sumf1 (Diez-Roux and Ballabio 2005); hence, it can be viewed as the master regulator of proteoglycan desulfation. Further underscoring the biological importance of Sumf1 and of proteoglycan desulfation a broad spectrum of diseases, most of them including skeletal abnormalities, results from loss of function mutations in various sulfatases or in Sumf1 itself (Diez-Roux and Ballabio 2005). In most of these diseases, called mucopolysaccharidoses, the lack of lysosomal sulfatases causes a block in GAG degradation and an intralysosomal GAG accumulation (Neufeld and Muenzer 2001).

In order to define the importance of proteoglycan desulfation during skeletogenesis we studied mice lacking *Sumf1* (Settembre et al. 2007). We show here that although *Sumf1* is expressed in osteoblasts during skeletogenesis, it has no overt function in osteoblasts. In contrast, *Sumf1* and, as a result, proteoglycan desulfation, favors ECM production, chondrocyte autophagy, and promotes chondrocyte proliferation and differentiation. Genetic and biochemical evidence show that *Sumf1* regulation of chondrocyte proliferation and differentiation occurs by inhibiting FGF signaling.

## Results and Discussion

Sumf1 expression during skeletogenesis

To determine whether *Sumf1* is an appropriate tool to study the role(s) of proteoglycan desulfation during skeletogenesis we examined its pattern of expression using a *LacZ* gene inserted in the *Sumf1* locus (Settembre et al. 2007). β-Galactosidase staining of whole embryos showed that *Sumf1* starts to be expressed in skeletal elements as early as embryonic day 14.5 (E14.5) (Fig. 1A–C). Histological analysis revealed that this staining was
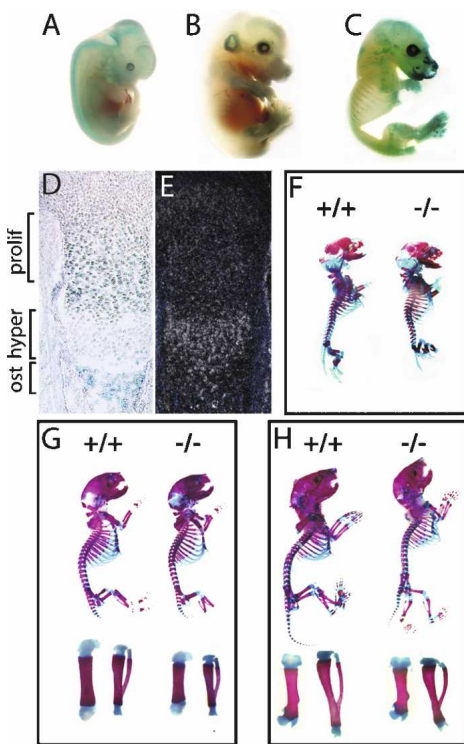
Figure 1. Skeletal development in *Sumf1*⁻/⁻ embryos and mice. (*A–C*) X-gal-stained *Sumf1*⁺/⁻ E12.5, E14.5, and E16.5 embryos. *LacZ* was expressed in all cartilagineous elements starting at E14.5. (*D*) Femoral growth plate section showing *LacZ* staining in proliferating chondrocytes and osteoblasts. (*E*) In situ hybridization of *Sumf1* in E16.5 growth plate. (*F–H*) Alcian blue/alizarin red staining of E16.5 (*F*), newborn (*G*), and P4 (*H*) wild-type and *Sumf1*⁻/⁻ embryos and mice. (*G,H*) Femur and tibia magnification of newborn and P4 *Sumf1*⁻/⁻ and wild-type mice.

equally strong in proliferating chondrocytes of the growth plate and in osteoblasts of the developing bones, while staining in hypertrophic chondrocytes was weak (Fig. 1D); in situ hybridization analysis of *Sumf1* expression while confirming that *Sumf1* was expressed in proliferating chondrocytes and osteoblasts also revealed a strong expression in hypertrophic chondrocytes (Fig. 1E). Thus, *Sumf1* is a molecular marker of all cell types in the developing skeleton.

Proteoglycan desulfation affects endochondral ossification

As a first approach to study the importance of proteoglycan desulfation during skeletogenesis we analyzed skeletal preparations of *Sumf1*⁻/⁻ embryos and mice stained with alcian blue for unmineralized cartilagineous ECMs and alizarin red for mineralized cartilagineous and bony ECMs. E14.5 *Sumf1*⁻/⁻ embryos were indistinguishable from wild-type littermates (data not shown), an observation consistent with the fact that *Sumf1* is not express in the developing skeleton before this stage. In contrast, examination of E16.5 embryos revealed that femurs were significantly shorter in *Sumf1*⁻/⁻ than in wild-type embryos (3.0 mm wild type vs. 2.8 mm *Sumf1*⁻/⁻; $n = 3$, $P < 0.05$). This dwarfism, that worsened in newborn *Sumf1*⁻/⁻ mice (4.2 mm wild type vs. 3.8 mm *Sumf1*⁻/⁻; $n = 5$, $P < 0.01$) was generalized, as every bone analyzed

(femurs, vertebrae, mandibles, components of the skull) were shorter in *Sumf1*⁻/⁻ than in wild-type littermates (Fig. 1F,G; data not shown). These morphological abnormalities that were not caused by a decrease in insulin growth factor 1 production (Supplemental Fig. 1A), illustrate that the extent of proteoglycan desulfation is an important determinant of the growth of most skeletal elements during embryonic development.

### Proteoglycan desulfation affects growth plate cellularity

Next, we performed histological and gene expression analyses. Consistent with the absence of morphological defects in E14.5 *Sumf1*⁻/⁻ embryos there was no difference in the morphology of the various populations of chondrocytes between *Sumf1*⁻/⁻ and wild-type embryos at that stage and expression of α_1(II) *Collagen*, a marker of proliferating chondrocytes, and of α_1(X) *Collagen*, a marker of hypertrophic chondrocytes, was similar in *Sumf1*⁻/⁻ and wild-type embryos at E14.5 (Fig. 2A). At E16.5 the zone of proliferating chondrocytes, determined by cell morphology and the extent of α_1(II) *Collagen* expression, was significantly shorter in *Sumf1*⁻/⁻ than in wild-type embryos (Fig. 2B). The intensity of α_1(X) *Collagen* expression was also weaker in E16.5 *Sumf1*⁻/⁻ than in wild-type embryos (Fig. 2B). At birth, the classical columnar organization of the proliferating chondrocytes was lost in the *Sumf1*⁻/⁻ growth plate and expression of α_1(II) *Collagen* and of α_1(X) *Collagen* was again noticeably weaker in mutant than in wild-type growth plate (Fig. 2C). Remarkably, despite the high level of *Sumf1* expression in osteoblasts, bone formation defined by the presence of bone trabeculae and expression of α_1(I) *Collagen* was not affected by *Sumf1* inactivation (Fig. 2B,C,D). Taken together, these results suggest that inactivation of *Sumf1*—i.e., inhibition of proteoglycan desulfation—affects more severely chondrogenesis than osteogenesis during development.

We next investigated whether the weaker expression of α_1(II) and α_1(X) *Collagen* in the *Sumf1*⁻/⁻ growth plate was reflecting a decrease in chondrocyte number. We observed that there was a significant decrease in chondrocyte number in *Sumf1*⁻/⁻ growth plates at E16.5 and at postnatal day 0 (P0) (Fig. 3A), indicating that the extent of proteoglycan desulfation regulates, in ways studied below, chondrocyte survival and/or proliferation during endochondral ossification.

Proteoglycan desulfation influences ECM production and turnover

Since abnormalities in proteoglycan desulfation result in lysosomal defects in several tissues in vertebrates (Neufeld and Muenzer 2001), we asked if lysosomal function was affected in *Sumf1*⁻/⁻ chondrocytes and/or osteoblasts.

Electron microscopic (EM) analysis of wild-type and mutant growth plates did not reveal any morphological difference between *Sumf1*⁻/⁻ and wild-type chondrocytes at E14.5 (Fig. 3C), while in E16.5 *Sumf1*⁻/⁻ embryos and newborn mice the cytoplasm of chondrocytes was filled with vacuolar structures that had characteristic appearance of lysosomes filled with GAGs as seen in cells from patients affected by mucopolysaccharidoses (Neufeld and Muenzer 2001) (Fig. 3D,E, inset; Supplemental Fig.
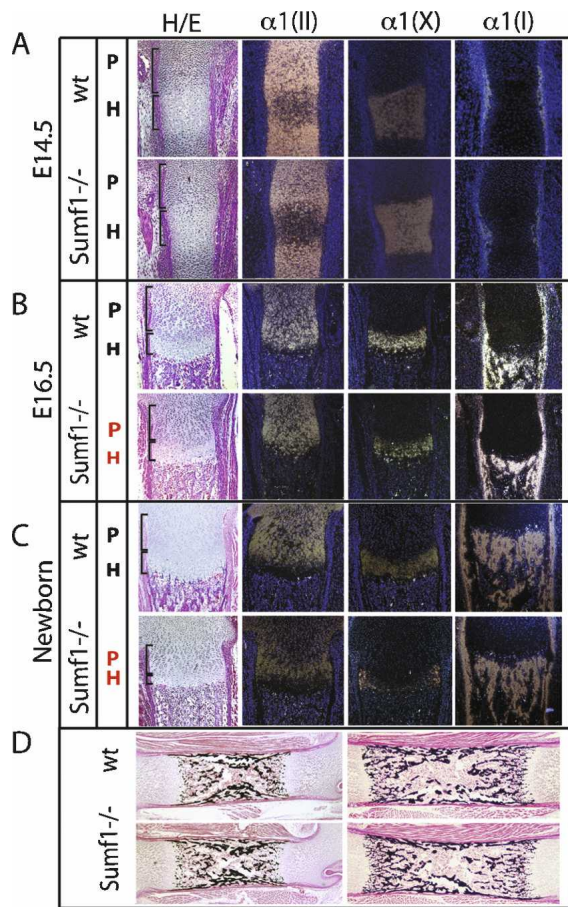
Figure 2. $Sumf1^{-/-}$ deficiency affects chondrogenesis but not osteogenesis. (*A*) Hematoxylin/eosin (H/E) staining and in situ hybridization analysis of femoral sections. No differences between $Sumf1^{-/-}$ and wild-type growth plates are observed in E14.5 embryos, while in E16.5 (*B*) and newborn (*C*) there is a progressive shortening of both proliferative and hypertrophic area in mutant mice. Expression of $\alpha 1(II)$ and $\alpha 1(X)$ *Collagen* was decreased in newborn $Sumf1^{-/-}$ compared with wild-type mice. $\alpha 1(I)$ *Collagen* expression was similar in wild-type and $Sumf1^{-/-}$ samples at all stages analyzed. (*D*) Von Kossa-Van Gieson staining of femoral sections showed normal mineralization (black staining) in E16.5 and newborn $Sumf1^{-/-}$ mice. Magnification: *A–C*, 100×, *D*, 50×.

1B). Lysosomal vacuolization in osteoblasts was not nearly as dramatic, further suggesting that proteoglycan desulfation is a more important process during chondrogenesis than osteogenesis (Fig. 3F).

This impaired lysosomal degradation of GAGs in chondrocytes led us to ask whether the amount of GAGs present in the ECM was affected by the absence of *Sumf1* in chondrocytes by measuring the amount of GAGs in wild-type and in $Sumf1^{-/-}$ chondrocytes. As hypothesized, mutant chondrocytes produced 30% fewer membrane-bound GAGs than wild-type chondrocytes; GAG secretion in the medium was also reduced by 50% (Fig. 3G). This explained why staining with alcian blue, a dye marking proteoglycans, was significantly weaker in $Sumf1^{-/-}$ than in wild-type growth plates (Fig. 3H). EM analysis of ER and Golgi did not shown any morphological differences between wild-type and $Sumf1^{-/-}$ chondrocytes at E16.5 or P0, indicating that the reduced amount of ECM is not caused by a block in the GAGs biosynthetic pathway (Supplemental Fig. 1C).

## Chondrocyte autophagy is hampered in the absence of proteoglycan desulfation

We next asked what were the mechanisms leading the decrease in chondrocyte number in $Sumf1^{-/-}$ growth plate. There was no overt increase in chondrocyte apoptosis in $Sumf1^{-/-}$ mice before or after birth (data not shown). BrdU incorporation did not reveal any significant difference in chondrocyte proliferation between $Sumf1^{-/-}$ and wild-type embryos at E14.5 and E16.5 either, although chondrocyte proliferation was significantly decreased after birth in $Sumf1^{-/-}$ mice (Fig. 4A). Thus, at least two mechanisms explain the paucity of chondrocytes in the $Sumf1^{-/-}$ growth plate: a decrease in chondrocyte proliferation beyond birth, and another one, yet to be determined, during embryonic development.

Macroautophagy (hereafter referred as autophagy) is a lysosomal process of cellular self-digestion required for turnover of cytoplasmic structure and for producing energy in conditions of cellular starvation (Mizushima 2007). Since chondrocytes reside in a hypoxic and avascular environment (Schipani et al. 2001) we hypothesized that autophagy may be a physiological means al-
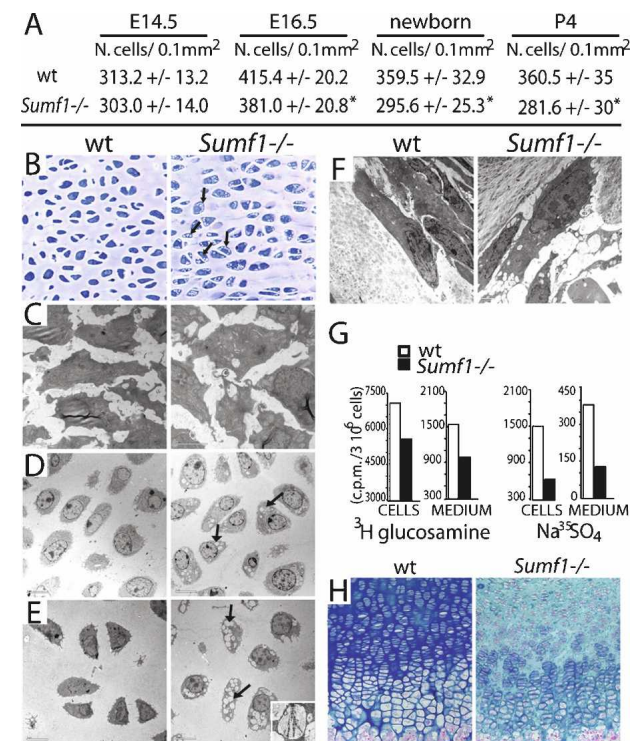
Figure 3. Defective ECM in $Sumf1^{-/-}$ growth plate. (*A*) Chondrocyte number in the growth plate proliferative zone. Values are the mean ± SD. Student's test (*) $P < 0.05$. (*B*) Toluidin blue staining of chondrocostal cartilage of newborn $Sumf1^{-/-}$ and wild-type littermates. Note the presence of cytoplasmatic vacuolization in $Sumf1^{-/-}$ chondrocytes. (*C*) EM analysis of E14.5 $Sumf1^{-/-}$ and wild-type chondrocytes showing no evidence of lysosomal vacuolization in $Sumf1^{-/-}$ embryos. (*D,E*) Cytoplasmatic vacuoles in E16.5 (*D*) and newborn (*E*) $Sumf1^{-/-}$ chondrocytes filled with amorphous material (GAGs) and partially degraded collagen fiber (*E, inset*). (*F*) $Sumf1^{-/-}$ osteoblasts are less affected than chondrocytes by lysosomal vacuolization. (*G*) ECM proteoglycan produced by $Sumf1^{-/-}$ and wild-type primary chondrocytes labeled with ³H-glucosamine and Na³⁵SO₄. ECM amount was estimated by ³H and ³⁵S incorporation and normalized for cells number. (*H*) Decreased alcian blue staining in P7 $Sumf1^{-/-}$ compared with wild-type growth plate.
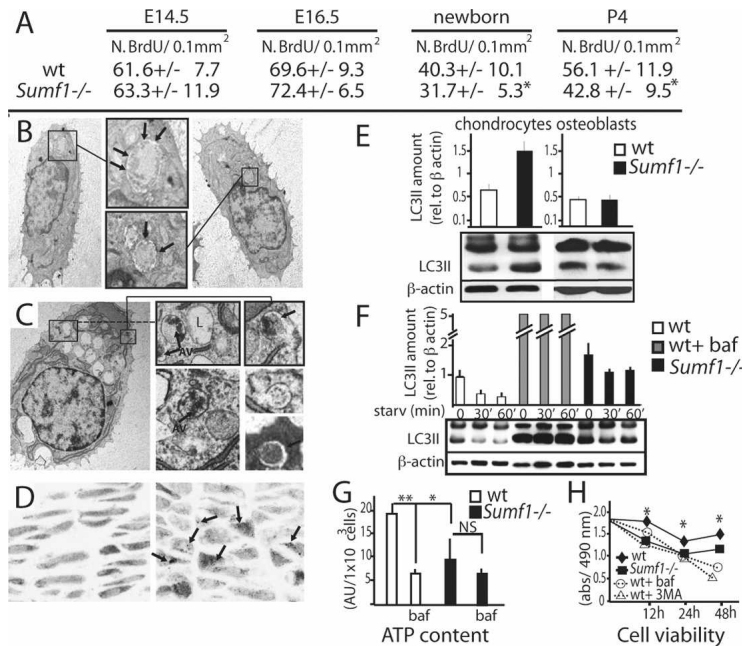
Figure 4. Abnormal autophagy in *Sumf1*$^{-/-}$ chondrocytes. (*A*) Number of BrdU positive cells present in the proliferative zone of the growth plate. Values are the mean ± SD. Student's test (*) $P < 0.05$. (*B*) EM analysis of newborn chondrochostal cartilage revealed the presence of autophagosomes in wild-type chondrocytes. (Boxed *inset*) Note the double membrane vesicles surrounding a portion of cytoplasm (arrows). (*C*) Chondrocyte from newborn *Sumf1*$^{-/-}$ showing more autophagosomes (AV) surrounded by enlarged lysosomes (L). (*D*) Confocal microscopy analysis of *Sumf1*$^{-/-}$;GFP-LC3 and wt;GFP-LC3 growth plate. In GFP-LC3 chondrocytes (*left*) the GFP fluorescence was more diffused throughout the cytoplasm while in *Sumf1*$^{-/-}$;GFP-LC3 it was aggregated in cytoplasmatic dots (*right*). (*E*) Western blot analysis showing a 2.5-fold increase in LC3II level in newborn *Sumf1*$^{-/-}$ chondrocytes. No difference was observed in osteoblasts. Values shown are means of triplicate experiments. (*F*) Abnormal autophagy in *Sumf1*$^{-/-}$ chondrocytes during serum and nutrient starvation. Wild-type and *Sumf1*$^{-/-}$ chondrocytes were starved for the indicated period of time, harvested, and subjected to LC3 immunoblotting. *Sumf1*$^{-/-}$ chondrocytes and wild type stimulated with Baf presented an increased amount of LC3II compared with wild-type chondrocytes at all time points analyzed. (*G*) ATP amount is decreased in wild-type chondrocytes when autophagy is inhibited with Baf. *Sumf1*$^{-/-}$ chondrocytes displayed a lower level of ATP compared with wild-type and Baf treatment did not affected significantly ATP concentration. (*H*) Wild-type and *Sumf1*$^{-/-}$ chondrocytes were cultured in serum and glucose-free medium for 2 d. Wild-type chondrocytes were also treated with baf and 3-methyladenine, another inhibitor of autophagy. Cell viability was monitored after 12 h, 24 h, and 48 h. Error bars represent SEM. Student's test (*) $P < 0.05$; (**)$P < 0.01$.

lowing them to survive, and that impairment in this process could cause chondrocyte death in *Sumf1*$^{-/-}$ embryos. Indeed, EM analysis of E16.5 embryos and newborn wild-type mice identified vacuoles containing a portion of cytoplasm and degraded organelles, a feature characteristic of autophagy (Mizushima 2007) (Fig. 4B, arrows). Remarkably, the number of autophagosome vacuoles (autophagosomes) was greatly increased in mutant chondrocytes (Fig. 4C).

To establish rigorously that the number of autophagosomes was increased in *Sumf1*$^{-/-}$ chondrocytes we used in vivo and biochemical approaches. First, we generated *Sumf1*$^{-/-}$ mice harboring in all cells a transgene expressing a GFP-tagged LC3 protein (Mizushima et al. 2004). During autophagy the free cytoplasmatic LC3I isoform of the LC3 protein is converted into LC3II, a specific marker of autophagosomes (Kabeya et al. 2000). Consistent with an increase in the number of autophagosomes

GFP immunoreactivity was noticeably stronger in *Sumf1*$^{-/-}$;GFP-LC3 than in *GFP-LC3* chondrocytes (Supplemental Fig. 1D). Moreover, while the GFP fluorescence was distributed throughout the cytoplasm in wild-type chondrocytes, it was clustered in autophagosomes in *Sumf1*$^{-/-}$;GFP-LC3 chondrocytes (Fig. 4D, arrows). Western blot quantification of LC3II proteins showed also a 2.5-fold increase in *Sumf1*$^{-/-}$ compared with wild-type growth plates (Fig. 4E). This was not observed in *Sumf1*$^{-/-}$ osteoblasts (Fig. 4E), further illustrating that these cells are not overtly influenced by the absence of *Sumf1*.

Since GAGs accumulation can inhibit lysosomal function (Li et al. 2004), we tested whether this increase in the number of autophagosomes in *Sumf1*$^{-/-}$ chondrocytes was reflecting a failure of lysosome to digest autophagosomes by treating wild-type and *Sumf1*$^{-/-}$ chondrocytes with bafilomycin (Baf), an inhibitor of the autophagosome–lysosome fusion (Yamamoto et al. 1998), a necessary step during autophagy (Mizushima 2007). Baf treatment of wild-type chondrocytes triggered an increase in autophagosome accumulation as measured by LC3-II level and a significant decrease of energy (ATP) production (Fig. 4F,G). In contrast, in *Sumf1*$^{-/-}$ chondrocyte the level of LC3II was higher than in wild-type chondrocytes, and Baf did not decrease energy production significantly (Fig. 4F,G). These results support the notion that autophagy is impaired in *Sumf1*$^{-/-}$ chondrocytes. To determine if this impairment of autophagy could lead to chondrocyte death, wild-type and mutant chondrocytes were cultured in glucose-free and serum-free medium, a condition in which autophagy is required for energy production and cell survival (Lum et al. 2005). LC3-II immunoreactivity decreased rapidly upon nutrient starvation, suggesting that autophagosomes were efficiently digested by lysosomes in wild-type but not in *Sumf1*$^{-/-}$ chondrocytes (Fig. 4F). Moreover, when measured with colorimetric assay, cell viability was decreased in *Sumf1*$^{-/-}$ chondrocytes compared with wild-type cells (Fig. 4H).

In summary, in vivo and cell-based assays establish that autophagy is used by wild-type chondrocytes to produce energy and suggest that disruption of intralysosomal GAG digestion impairs autophagy in *Sumf1*$^{-/-}$ chondrocytes. This impairment in turn leads to cell death.

Proteoglycan desulfation regulates chondrocyte proliferation and differentiation

To explain the decrease in chondrocyte proliferation noted in *Sumf1*$^{-/-}$ mice we asked whether *Sumf1* expression modulates growth factor signaling. We first looked at Indian Hedgehog (Ihh), since its signaling is influenced in vivo by GAGs (Koziel et al. 2004), but failed to detect any difference in the level of expression of *Ihh* or of its receptor *Patched* (*Ptch*) between *Sumf1*$^{-/-}$ and wild-type chondrocytes at E16.5, P0, or P4 (Supplemental Fig. 2; data not shown). These observations indicate that *Sumf1* deletion does not affect overtly *Ihh* signaling during endochondral ossification.
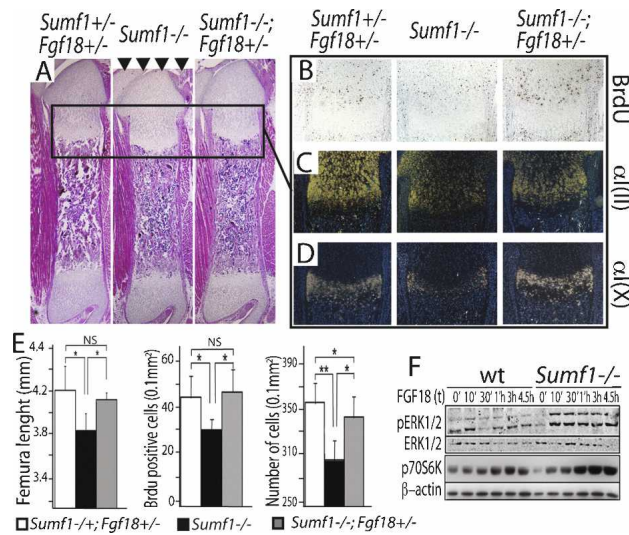
Figure 5. *Sumf1* regulates FGF18 activity during endochondral ossification. (*A*) H/E staining of femurs showing shortening of *Sumf1*$^{-/-}$ bone length (arrowheads) and its rescue in *Sumf1*$^{-/-}$;*fgf18*$^{+/-}$ mice. Brdu staining (*B*) and in situ expression of α1(II) Collagen (*C*) and α1(X) Collagen (*D*) in wild-type (*left*), *Sumf1*$^{-/-}$ (*middle*), and *Sumf1*$^{-/-}$;*fgf18*$^{+/-}$ mice. (*E*) Quantification of femoral length, BrdU index, and cell number in newborn wild-type, *Sumf1*$^{-/-}$, and *Sumf1*$^{-/-}$;*fgf18*$^{+/-}$ mice. At least three mice were analyzed per each genotype. Error bars represent SEM. Student's test (*) $P < 0.05$; (**) $P < 0.01$. (*F*) Primary chondrocytes from wild-type and *Sumf1*$^{-/-}$ mice treated with Fgf18 (20 ng/mL) for the indicated period of time. Note the more sustained phosophorylation of ERK and 70S6k in *Sumf1*$^{-/-}$ than in wild-type chondrocytes following Fgf18 treatment.

The defect in chondrocyte proliferation and differentiation noticed in *Sumf1*$^{-/-}$ mice was reminiscent of what is observed in mice harboring an increase in FGF signaling (Ornitz and Marie 2002). To determine if FGF signaling was increased we generated *Sumf1*$^{-/-}$ mice lacking one copy of *Fgf18*, a known regulator of chondrocyte proliferation (Liu et al. 2002). Several lines of evidence indicate that proteoglycan desulfation indeed regulates, directly or indirectly, FGF signaling.

First, skeletal preparation showed that removing one allele of *Fgf18* rescued *Sumf1*$^{-/-}$ mice short stature at P0 as determined by femur length (Fig. 5A [arrowhead], quantification in E). Second, chondrocyte proliferation measured by BrdU incorporation, which was reduced by 20% in the P0 *Sumf1*$^{-/-}$, was indistinguishable from wild-type in *Sumf1*$^{-/-}$;*Fgf18*$^{+/-}$ P0 mice (Fig. 5B, quantification in E). As a result, the zone of hypertrophic chondrocytes was larger, and there was a significant increase of growth plate cellularity in *Sumf1*$^{-/-}$;*Fgf18*$^{+/-}$ compared with *Sumf1*$^{-/-}$ mice (Fig. 5E). Third α$_1$(II) Collagen and α$_1$(X) Collagen expression was restored almost to its normal intensity in *Sumf1*$^{-/-}$;*Fgf18*$^{+/-}$ newborn mice (Fig. 5C,D). Together, these data support the notion that the absence of *Sumf1* results in an increase in FGF signaling, explaining the decrease in chondrocyte proliferation observed in *Sumf1*$^{-/-}$ mice. Of note, removing one Fgf18 allele did not normalize the chondrocyte number (Fig. 5E), indicating that the abnormal autophagy seen in the Sumf1$^{-/-}$ embryos is not secondary to the increase in FGF signaling.

To identify the cause(s) of this increased FGF signaling in *Sumf1*$^{-/-}$ chondrocytes we first compared *Fgf18* expression in *Sumf1*$^{-/-}$ and wild-type newborn mice but

failed to detect any significant difference (Supplemental Fig. 3), thus ruling out that *Sumf1* is not a regulator of *Fgf18* expression. It has been proposed that proteoglycans, through their degree of sulfation, modulate the affinity of FGFs for their cognate receptors (Bishop et al. 2007). To determine if this was the case in *Sumf1*$^{-/-}$ mice we stimulated wild-type and *Sumf1*$^{-/-}$ primary chondrocytes with FGF18 and measured activation of FGF signaling. Both the ERK kinase and the ribosomal protein S6, which are involved in FGF signaling (Murakami et al. 2004), were more phosphorylated in *Sumf1*$^{-/-}$ than in wild-type chondrocytes (Fig. 5F). These results support the hypothesis that *Sumf1*, and more generally proteoglycan desulfation, influence FGF signaling in chondrocytes during skeletal development.

Sulfatases catalyze desulfation of the GAGs moiety of proteoglycans in the intracellular and extracellular space. These enzymes are substrates of *Sumf1* whose only known function is to activate sulfatases. By studying endochondral ossification in *Sumf1*$^{-/-}$ mice we show that proteoglycan desulfation regulates several aspects of chondrocyte biology. Indeed, the block in proteoglycan desulfation caused by *Sumf1*$^{-/-}$ deletion severely decreases chondrocytes viability by hampering their capacity to generate enough energy through autophagy to survive in their avascular environment. Our data suggest that this defect in autophagy is caused, in part, by the engulfment of lysosomes by undigested GAGs that leads to an impairment of the autophagosome–lysosome fusion (Settembre et al. 2008).

Growth plate cartilage is a hypoxic structure in which the transcription factor *Hif1*α is required for chondrocytes survival. The fact that hypoxia stimulate autophagy (Mizushima 2007) suggests that autophagy could be one pathway through which *Hif1*α allows chondrocytes to survive. Moreover, chondrocytes starvation does not increase the autophagosome number as commonly observed in other cellular lines, suggesting that in this cell type autophagy is a constitutive rather than an adaptive pathway.

Our study also shows that proteoglycan desulfation is a negative regulator of *Fgf18* signaling during endochondral ossification. This may be a direct consequence of the level of desulfation of certain proteoglycans such as heparan sulphate proteoglycan (HSPG) rather than a secondary effect of the engulfment of lysosomes with GAGs. Our results are in agreement with the notion that, in cell culture, ECM proteoglycans, and particularly HSPG bind members of FGF family (Bishop et al. 2007). It is likely that the two main sulfatases involved in this regulatory pathways are *Sulf1* and *Sulf2*, which are strongly expressed in chondrocytes and whose substrate is HSPG (Lum et al. 2007). Together, our data show that proteoglycan desulfation eventually affects several aspects of chondrocyte biology during skeletal development not only by determining lysosomal function but also by modulating growth factor signaling.

Materials and methods

Animals

Skeletal preparation
Skeletons were fixed in 100% ethanol overnight and stained with alcian blue and alizarin red according to standard protocols. At least three mice of each genotype were analyzed per stage.

LacZ staining and BrdU labeling
Embryos were fixed in 1% paraformaldehyde, 0.2% glutalaldehyde, and stained overnight with X-Gal (Roche). Specimens were embedded in paraffin, sectioned at 6 μm, and counterstained with eosin (Sigma). Embryos were labeled with BrdU by injecting pregnant females with 500 μL of 10 mM BrdU 1 h before harvest. BrdU incorporation was detected using a Zymed BrdU staining kit (Invitrogen). Three to five embryos/mice were analyzed for each genotype and age group. Statistical significance was assessed by the Student's test. ([*] $P < 0.05$; [**] $P < 0.01$).

Light and electron microscopy
Tibia were fixed in a 4% paraformaldehyde/1% glutaraldehyde fixative solution (pH 7.4), in 0.1 M sodium cacodylate buffer, dehydrated in ethanol, and embedded in plastic resin. Ultrathin sections (80 nm) were examined with a transmission electron microscope operated at 80 kV.

Cell cultures, Western blot analyses, and in situ hybridization
See the Supplemental Material.

Isolation of radiolabeled GAGs
Primary chondrocytes were cultured in presence of 10 μCi/mL $Na^{35}SO_4$ (25–40 Ci/mg) and 20 μCi/mL D-[6-^{3}H]glucosamine (40 Ci/mmol) for 3 d in sulfate and glucose-free medium. GAG extraction, purification, and analysis were performed as described previously (Bame and Esko 1989).

Acknowledgments

References

Bame, K.J. and Esko, J.D. 1989. Undersulfated heparan sulfate in a Chinese hamster ovary cell mutant defective in heparan sulfate N-sulfotransferase. *J. Biol. Chem.* **264:** 8059–8065.

Bishop, J.R., Schuksz, M., and Esko, J.D. 2007. Heparan sulphate proteoglycans fine-tune mammalian physiology. *Nature* **446:** 1030–1037.

Bulow, H.E. and Hobert, O. 2006. The molecular diversity of glycosaminoglycans shapes animal development. *Annu. Rev. Cell Dev. Biol.* **22:** 375–407.

Cosma, M.P., Pepe, S., Annunziata, I., Newbold, R.F., Grompe, M., Parenti, G., and Ballabio, A. 2003. The multiple sulfatase deficiency gene encodes an essential and limiting factor for the activity of sulfatases. *Cell* **113:** 445–456.

Diez-Roux, G. and Ballabio, A. 2005. Sulfatases and human disease. *Annu. Rev. Genomics Hum. Genet.* **6:** 355–379.

Esko, J.D. and Selleck, S.B. 2002. Order out of chaos: Assembly of ligand binding sites in heparan sulfate. *Annu. Rev. Biochem.* **71:** 435–471.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y., and Yoshimori, T. 2000. LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *EMBO J.* **19:** 5720–5728.

Koziel, L., Kunath, M., Kelly, O.G., and Vortkamp, A. 2004. Ext1-dependent heparan sulfate regulates the range of Ihh signaling during endochondral ossification. *Dev. Cell* **6:** 801–813.

Kronenberg, H.M. 2003. Developmental regulation of the growth plate. *Nature* **423:** 332–336.

Li, Z., Yasuda, Y., Li, W., Bogyo, M., Katz, N., Gordon, R.E., Fields, G.B., and Bromme, D. 2004. Regulation of collagenase activities of human cathepsins by glycosaminoglycans. *J. Biol. Chem.* **279:** 5470–5479.

Liu, Z., Xu, J., Colvin, J.S., and Ornitz, D.M. 2002. Coordination of chondrogenesis and osteogenesis by fibroblast growth factor 18. *Genes & Dev.* **16:** 859–869.

Lum, J.J., Bauer, D.E., Kong, M., Harris, M.H., Li, C., Lindsten, T., and Thompson, C.B. 2005. Growth factor regulation of autophagy and cell survival in the absence of apoptosis. *Cell* **120:** 237–248.

Lum, D.H., Tan, J., Rosen, S.D., and Werb, Z. 2007. Gene trap disruption of the mouse heparan sulfate 6-O-endosulfatase gene, Sulf2. *Mol. Cell. Biol.* **27:** 678–688.

Mizushima, N. 2007. Autophagy: Process and function. *Genes & Dev.* **21:** 2861–2873.

Mizushima, N., Yamamoto, A., Matsui, M., Yoshimori, T., and Ohsumi, Y. 2004. In vivo analysis of autophagy in response to nutrient starvation using transgenic mice expressing a fluorescent autophagosome marker. *Mol. Biol. Cell* **15:** 1101–1111.

Murakami, S., Balmes, G., McKinney, S., Zhang, Z., Givol, D., and de Crombrugghe, B. 2004. Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. *Genes & Dev.* **18:** 290–305.

Neufeld, E. and Muenzer, J. 2001. *The metabolic and molecular bases of inherited disease.* McGraw-Hill, New York.

Olsen, B.J., Reginato, A.M., and Wang, W. 2000. Bone development. *Annu. Rev. Cell Dev. Biol.* **16:** 191–220.

Ornitz, D.M. and Marie, P.J. 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes & Dev.* **16:** 1446–1465.

Perrimon, N. and Hacker, U. 2004. Wingless, hedgehog and heparan sulfate proteoglycans. *Development* **131:** 2509–2511.

Schipani, E., Ryan, H.E., Didrickson, S., Kobayashi, T., Knight, M., and Johnson, R.S. 2001. Hypoxia in cartilage: HIF-1α is essential for chondrocyte growth arrest and survival. *Genes & Dev.* **15:** 2865–2876.

Settembre, C., Annunziata, I., Spampanato, C., Zarcone, D., Cobellis, G., Nusco, E., Zito, E., Tacchetti, C., Cosma, M.P., and Ballabio, A. 2007. Systemic inflammation and neurodegeneration in a mouse model of multiple sulfatase deficiency. *Proc. Natl. Acad. Sci.* **104:** 4506–4511.

Settembre, C., Fraldi, A., Jahreiss, L., Spampanato, C., Venturi, C., Medina, D., de Pablo, R., Tacchetti, C., Rubinsztein, D.C., and Ballabio, A. 2008. A block of autophagy in lysosomal storage disorders. *Hum. Mol. Genet.* **17:** 119–129.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., and Tashiro, Y. 1998. Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Struct. Funct.* **23:** 33–42.