Runx2 inhibits chondrocyte proliferation and hypertrophy through its expression in the perichondrium

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The perichondrium, a structure made of undifferentiated mesenchymal cells surrounding growth plate cartilage, regulates chondrocyte maturation through poorly understood mechanisms. Analyses of loss- and gain-of-function models show that Twist-1, whose expression in cartilage is restricted to perichondrium, favors chondrocyte maturation in a Runx2-dependent manner. Runx2, in turn, enhances perichondrial expression of *Fgf18*, a regulator of chondrocyte maturation. Accordingly, compound heterozygous embryos for *Runx2* and *Fgf18* deletion display the same chondrocyte maturation phenotype as *Fgf18*-null embryos. This study identifies a transcriptional basis for the inhibition of chondrocyte maturation by perichondrium and reveals that Runx2 fulfills antagonistic functions during chondrogenesis.

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Endochondral bone formation is a complex process that involves several cell types with distinct patterns of gene expression. The initial step in this process is characterized by the aggregation of undifferentiated mesenchymal cells expressing α*1(I)* and α*1(III) Collagen* into condensations at the location and with the overall shape of future skeletal elements. Subsequently, cells within these condensations differentiate into chondrocytes that do not express any more α*1(I) Collagen* but instead, and among other genes, $\alpha 1(II)_b$ *Collagen*. Cartilage anlagen then enlarge through proliferation of chondrocytes that elongate to form prehypertrophic chondrocytes that will eventually exit the cell cycle to become bona fide hypertrophic chondrocytes. Genetically, hypertrophic chondrocytes differ from proliferating chondrocytes as they express α*1(X) Collagen* but not α*1(II) Collagen* (Kronenberg 2003). Throughout skeletogenesis, layers of α*1(I) Collagen*-expressing undifferentiated mesenchymal cells persist and surround cartilage anlagen to form a structure called the perichondrium (Kronenberg 2003).

The transcriptional control of cell differentiation during skeletogenesis has been a topic of intense studies in the last 10 yr, leading to the identification of several key genes. For instance, *Sox9* along with *Sox5* and *Sox6* are seen as the main transcription factors triggering mesenchymal condensations and initiating chondrocyte differentiation (Lefebvre et al. 1998; Bi et al. 1999; Smits et al. 2001). On the other hand, *Runx2* appears to be the earliest transcriptional determinant of osteoblast differentiation (Ducy et al. 1997; Komori et al. 1997; Otto et al. 1997). Runx2 has broader functions during skeletogenesis since it is, along with *Runx3,* an inducer of chondrocyte hypertrophy (Takeda et al. 2001; Ueta et al. 2001; Yoshida et al. 2004). This latter role of Runx2 is explained by its transient expression in prehypertrophic chondrocytes.

Runx2 is also expressed at high levels and throughout skeletogenesis in cells of the perichondrium, suggesting that it may have additional roles during chondrogenesis (Ducy et al. 1997). This hypothesis is further supported by the recognized influence exerted by the perichondrium on chondrocyte maturation defined here as chondrocyte proliferation and hypertrophy. Indeed, removal of the perichondrium from chicken tibia organ cultures results in an increase in chondrocyte proliferation and in a larger zone of chondrocyte hypertrophy (Long and Linsenmayer 1998; Di Nino et al. 2001), implying that a yet unknown genetic cascade takes place in perichondrium to inhibit chondrocyte maturation.

While studying the regulation of Runx2 osteogenic function, we observed that the nuclear protein Twist-1 inhibits it by binding to its DNA-binding domain through a novel domain, the Twist box (Bialek et al. 2004). Part of this demonstration relied on the use of a hypomorphic *Twist-1* allele called *Charlie Chaplin* (*CC/CC*) in which a missense mutation in the Twist box decreases Twist-1's ability to interact with Runx2. We show here that chondrocyte maturation is regulated by *Twist-1* although it is never expressed in chondrocytes during development. Instead, *Twist-1* expression is restricted to cells of the perichondrium, where it regulates the function of Runx2 that in turn controls expression of *Fgf18*, a negative regulator of chondrocyte maturation (Liu et al. 2002; Ohbayashi et al. 2002). This study proposes a genetic and molecular basis for the role exerted by the perichondrium on chondrocyte maturation. In addition, it demonstrates that Runx2 exerts a broader array of functions during chondrogenesis than initially thought.

Results and Discussion

Twist-1 *favors chondrocyte maturation*

With the original purpose of analyzing Twist-1 functions during osteoblast differentiation, we generated trans-

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genic embryos expressing, under the control of α*1(I) Collagen* regulatory elements that are active in mesenchymal cells (Rossert et al. 1995), an HA-tagged Twist-1 molecule or an HA-tagged truncated Twist-1 molecule containing Twist-1's N-terminal region (HA-Twist-1_N) (Fig. 1A). This latter form of Twist-1 contains its nuclear localization signal but does not affect Runx2 function (Bialek et al. 2004). These transgenic embryos were analyzed at embryonic day 15.5 (E15.5) and E16.5. Real-time PCR showed an ~1.5-fold increased expression of *Twist-1* and *Twist-1_N* in all founder transgenic embryos, and immunohistochemical study established that HA-Twist-1 and HA-Twist-1_N proteins were present in cells of the bone collar and perichondrium in ribs and humeri but not in chondrocytes of transgenic embryos (Fig. 1B,C; Supplementary Fig. 1A).

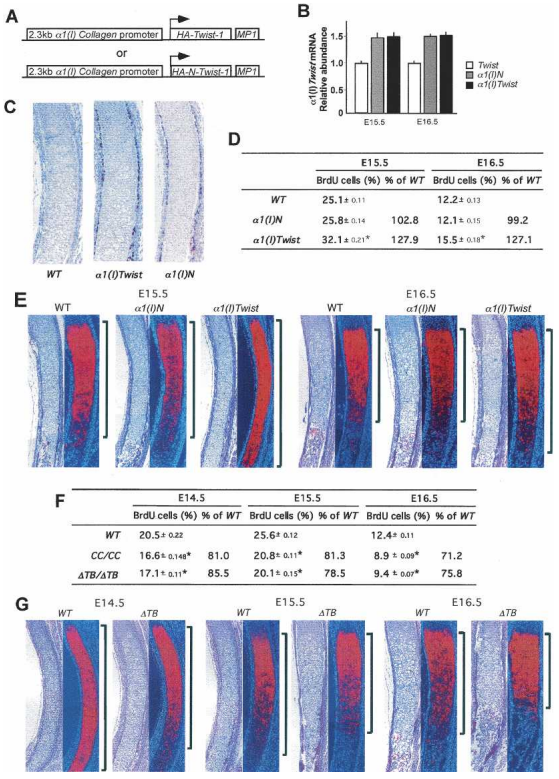

Figure 1. Twist-1 regulates chondrocyte maturation. (*A*) Schematic representation of the α*1(I) Collagen-Twist* and α*1(I) Collagen-Twist$_N$* constructs. (*B*) Real-time PCR analysis for *Twist-1* of wild-type (WT), α*1(I) Collagen-Twist*, and α*1(I) Collagen-Twist$_N$* expression. (*C*) Immunohistochemical analysis of ribs of wild-type, α*1(I) Collagen-Twist*, and α*1(I) Collagen-Twist$_N$* embryos using an anti-HA antibody. Immunoreactivity against HA is seen in perichondrium of α*1(I) Collagen-Twist* and α*1(I) Collagen-Twist$_N$* skeletal elements. (*D*) BrdU incorporation analysis in ribs of E15.5 and E16.5 wild-type, α*1(I) Collagen-Twist*, and α*1(I) Collagen-Twist$_N$* embryos. Increased chondrocyte proliferation in α*1(I) Collagen-Twist* but not in α*1(I) Collagen-Twist$_N$* embryos. (*E*) Histological analysis and in situ hybridization for α*1(X) Collagen* expression in ribs of E15.5 and E16.5 wild-type, α*1(I) Collagen-Twist*, and α*1(I) Collagen-Twist$_N$* embryos. Note the increase in the area of α*1(X) Collagen* expression in α*1(I) Collagen-Twist* embryos. (*F*) BrdU incorporation analysis in ribs of E14.5, E15.5, and E16.5 wild-type, *CC/CC*, and Δ*TB*$^{-/-}$ embryos. Note the decrease in chondrocyte proliferation in CC/CC and Δ*TB*$^{-/-}$ ribs. (*G*) Histological analysis and in situ hybridization for α*1(X) Collagen* expression in ribs of E14.5, E15.5, and E16.5 wild-type and Δ*TB*$^{-/-}$ embryos. Note the decreased expression of α*1(X) Collagen* in Δ*TB* ribs.

Remarkably, E16.5 α*1(I) Collagen*-HA-*Twist-1* embryos displayed a marked advance in chondrocyte maturation in both ribs and humeri (Fig. 1D,E; Supplementary Fig. 1B,C). This was characterized by a significant increase in chondrocyte proliferation measured by BrdU incorporation and by an increase of the extent of the zone of hypertrophic chondrocytes determined histologically and by α*1(X) Collagen* expression. In contrast, α*1(I) Collagen*-HA-*Twist-1$_N$* embryos did not show any abnormality in chondrocyte maturation (Fig. 1D,E; Supplementary Fig. 1B,C).

This observation was surprising since an overexpression of *Twist-1* should result in a decrease in the activity of Runx2, a factor inducing chondrocyte hypertrophy (Takeda et al. 2001; Ueta et al. 2001). Thus, to determine if this was revealing an important biological function of Twist-1, we turned our attention to two loss-of-function models. The first one, the *CC/CC* mouse, harbors a missense mutation in the Twist box that decreases Twist-1's ability to interact with Runx2 and possibly other proteins. The second one, generated through homologous recombination in embryonic stem (ES) cells, lacks the entire Twist-box domain (Δ*TB*$^{-/-}$) (Supplementary Fig. 2). Both mutant mouse strains displayed major patterning defects affecting fore- and hindlimbs (data not shown); therefore we restricted our analysis of chondrocyte maturation to the ribs that are normally patterned in these mutant embryos. BrdU incorporation showed that chondrocyte proliferation was decreased in *CC/CC* and Δ*TB*$^{-/-}$ embryos at all stages analyzed (Fig. 1F). Chondrocyte hypertrophy, whether it was analyzed histologically or through α*1(X) Collagen* expression, was also less advanced in Δ*TB*$^{-/-}$ compared with wild-type embryos (Fig. 1G). These results are the mirror image of what was observed in α*1(I) Collagen-Twist-1* embryos; thus, gain- and loss-of-function models concur to show that favoring chondrocyte maturation is a biological function of *Twist-1*.

Twist-1 *is not expressed and has no overt function in chondrocytes*

To elucidate how *Twist-1* could regulate chondrocyte maturation, we first analyzed its pattern of expression between E13.5 and E16.5 in developing ribs and limbs. At both stages, *Twist-1* expression could be detected in α*1(I) Collagen*-expressing cells of the bone collar and perichondrium but not in α*1(II) Collagen*-expressing proliferating chondrocytes or α*1(X) Collagen*-expressing hypertrophic chondrocytes (Fig. 2A; Supplementary Fig. 3).

That in situ hybridization failed to detect *Twist-1* expression in chondrocytes did not formally exclude that *Twist-1* could be expressed in these cells below the limit of detection of in situ hybridization and yet affect chondrocyte maturation. To address this possibility, we deleted *Twist-1* from nonhypertrophic chondrocytes by crossing mice harboring a floxed allele of *Twist-1* (*Twist-1^{flox}*) with α*1(II) Collagen-Cre* transgenic mice to obtain *Twist-1$_{ch^{-/-}}$* mice (Supplementary Fig. 4). The regulatory elements of the α*1(II) Collagen* gene used to express *Cre-recombinase* in chondrocytes are inactive in cells of the perichondrium (Takeda et al. 2001); thus, the α*1(II) Collagen-Cre* transgenic mouse can delete genes of interest from nonhypertrophic chondrocytes but not from perichondrial cells. Histological examination of developing ribs and limbs did not show any overt difference in the

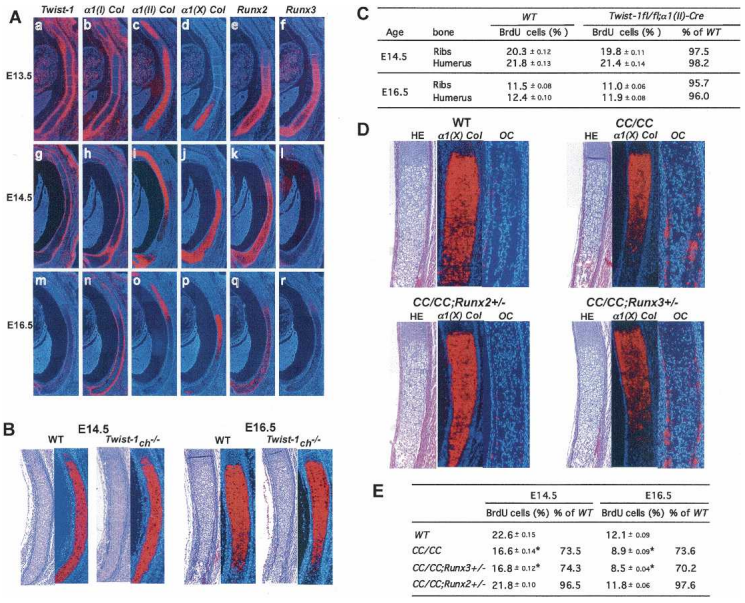

Figure 2. Twist-1 regulates Runx2 function in perichondrium. (*A*) In situ hybridization for *Twist-1* (panels *a*,*g*,*m*), α*1(I) Collagen* (panels *b*,*h*,*n*), α*1(II) Collagen* (panels *c*,*i*,*o*), α*1(X) Collagen* (panels *d*,*j*,*p*), *Runx2* (panels *e*,*k*,*q*), and *Runx3* (panels *f*,*l*,*r*) embryos at E13.5 (panels *a–f*), E14.5 (panels *g–l*), and E16.5 (panels *m–r*). *Twist-1* is coexpressed with *Runx2* in perichondrium and bone collar. (*B*) Histological analysis and in situ hybridization for α*1(X) Collagen* in ribs of E14.5 and E16.5 wild-type (WT) and α*1(II) Collagen-Cre; Twist-1*$^{flox/flox}$ embryos. No change was observed in α*1(X) Collagen* expression. (*C*) BrdU incorporation analysis in ribs and humeri of E14.5 and E16.5 wild-type and α*1(II) Collagen-Cre; Twist-1*$^{flox/flox}$ embryos. No change in BrdU uptake was observed. (*D*) Histological analysis and in situ hybridization for α*1(X) Collagen* and *Osteocalcin* expression in ribs of E16.5 wild-type, *CC/CC*, *CC/CC; Runx2*$^{+/-}$, and *CC/CC; Runx3*$^{+/-}$ embryos. The accelerated chondrocyte hypertrophy observed in *CC/CC* is rescued in *CC/CC; Runx2*$^{+/-}$ but not in *CC/CC; Runx3*$^{+/-}$ embryos. (*E*) BrdU incorporation analysis in ribs of E14.5 and E16.5 wild-type, *CC/CC*, *CC/CC; Runx2*$^{+/-}$, and *CC/CC; Runx3*$^{+/-}$ embryos. The decreased chondrocyte proliferation rate in *CC/CC* is normalized in *CC/CC; Runx2*$^{+/-}$ but not in *CC/CC; Runx3*$^{+/-}$ embryos.

extent of the zone of proliferating chondrocytes or in the onset of chondrocyte hypertrophy between *Twist-1*$_{ch}$$^{-/-}$ and wild-type embryos at E14.5 and E16.5. This was verified molecularly through the analysis of α*1(X) Collagen* expression and BrdU incorporation assays (Fig. 2B,C; Supplementary Fig. 4D). This absence of histological, cellular, or molecular abnormalities in mice harboring a chondrocyte-specific inactivation of *Twist-1* is in agreement with *Twist-1* expression studies and suggests that this gene influences chondrocyte proliferation through an indirect mechanism, presumably through its expression in perichondrial cells.

Runx2 *haploinsufficiency normalizes chondrocyte maturation in* CC/CC *embryos*

That the Twist box was required for Twist-1 regulation of chondrocyte maturation suggested that this process might occur through Twist-1 interaction with other nuclear proteins, possibly of the Runt family. To determine if this could be the case, we studied *Runx2* and *Runx3* expression in the perichondrium of developing ribs and limbs between E13.5 and E16.5. *Runx2* was more strongly expressed in α*1(I) Collagen*-expressing cells of the perichondrium, where *Twist-1* is also expressed, than in α*1(II) Collagen*-expressing chondrocytes throughout this period of development. In contrast, *Runx3* expression in perichondrial cells faded away beyond E13.5 (Fig. 2A; Supplementary Fig. 3).

To determine whether Twist-1 affects chondrocyte maturation through its interaction with Runx proteins in the perichondrium, we generated *CC/CC* embryos lacking one copy of either *Runx2* or *Runx3* and analyzed chondrogenesis in these compound mutant embryos. Remarkably, removing one allele of *Runx2* normalized chondrocyte proliferation measured by BrdU incorporation in E14.5 and E16.5 *CC/CC* ribs (Fig. 2E). Likewise, the extent of the hypertrophic chondrocyte zone, determined histologically and through α*1(X) Collagen* expression, was similar in *CC/CC; Runx2*$^{+/-}$ and wild-type embryos, while it was reduced in *CC/CC* embryos (Fig. 2D). Accordingly, osteoblast differentiation defined by *Osteocalcin* expression was delayed in *CC/CC; Runx2*$^{+/-}$ and wild-type compared with *CC/CC* embryos (Fig. 2D). In contrast, removing one allele of *Runx3* did not affect any cellular, histological, and molecular events in *CC/CC* ribs (Fig. 2D,E). This is in agreement with the absence of *Runx3* expression in perichondrium beyond E13.5. Taken together, these results provide genetic evidence suggesting that one mechanism whereby Twist-1 regulates chondrocyte maturation is through its ability to inhibit Runx2 function in perichondrial cells.

Fgf18 *as a mediator of Runx2 inhibition of chondrocyte maturation*

To elucidate how *Runx2*, through its perichondrial expression, inhibits chondrocyte maturation, we focused on genes expressed in perichondrial cells, harboring putative Runx2-binding sites and encoding secreted factors regulating chondrocyte maturation in vivo. Fgf18 is one gene fulfilling all these criteria (Liu et al. 2002; Ohbayashi et al. 2002).

The study of *Fgf18* expression in wild-type and various mouse mutant embryos with different levels of Runx2 activity supported the hypothesis that it may be a Runx2 target gene in perichondrial cells. Indeed, when compared with wild-type embryos, *Fgf18* expression in perichondrial cells of ribs and humeri was higher in E15.5 and E16.5 *CC/CC* and Δ*TB*$^{-/-}$ embryos, two mutant strains characterized by an increase in Runx2 activity, while it was decreased in *Runx2*$^{-/-}$ and α*1(I) Collagen*-HA-*Twist-1* embryos, which display a decrease in Runx2 activity (Fig. 3A; Bialek et al. 2004).

To provide a more compelling proof that *Fgf18* and *Runx2* interact in perichondrial cells to control chondrocyte maturation, we compared embryos heterozygous for both *Runx2* and *Fgf18* inactivation to *Fgf18*$^{-/-}$ embryos. Chondrocyte proliferation was nearly identical in E14.5 and E16.5 wild-type, *Runx2*$^{+/-}$, and *Fgf18*$^{+/-}$ embryos. In contrast, it was increased in *Runx2*$^{+/-}$*; Fgf18*$^{+/-}$ ribs and humeri to the same extent as in *Fgf18*$^{-/-}$ ribs and humeri (Fig. 3B). There was also in both *Runx2*$^{+/-}$*; Fgf18*$^{+/-}$ and in *Fg18*$^{-/-}$ embryos an increase of the area of hypertrophic chondrocytes determined by α*1(X) Collagen* expression and a delay in osteoblast differentiation, defined by *Osteocalcin* expression (Fig. 3C).

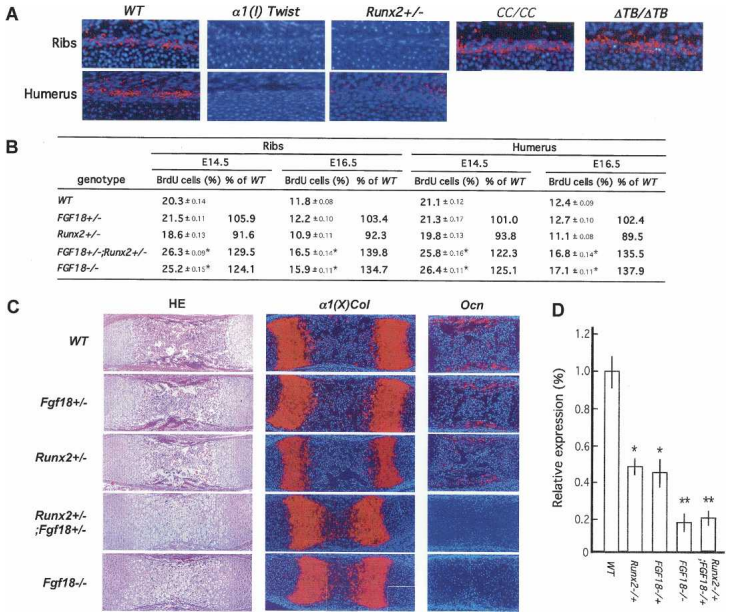

Figure 3. Runx2 regulates chondrocyte proliferation through *Fgf18*. (*A*) In situ hybridization analysis of *Fgf18* expression in ribs and humeri of E15.5 wild-type (WT), α*1(I) Twist-1*, *Runx2*$^{+/-}$, *CC/CC*, and Δ*Twist box*$^{-/-}$ embryos. Perichondrial *Fgf18* expression is decreased in α*1(I) Twist1* and *Runx2*$^{+/-}$ and increased in *CC/CC* and Δ*Twist box*$^{-/-}$ embryos. (*B*) BrdU incorporation analysis in ribs and humeri at E14.5 and E16.5 of wild-type, *Fgf18*$^{+/-}$, *Runx2*$^{+/-}$, *Fgf18*$^{+/-}$; *Runx2*$^{+/-}$, and *Fgf18*$^{-/-}$ embryos. Chondrocyte proliferation is similarly increased in *Fgf18*$^{+/-}$; *Runx2*$^{+/-}$ and *Fgf18*$^{-/-}$ embryos. (*C*) Histological and in situ hybridization analysis of α*1(X) Collagen* and *Osteocalcin* expression in humeri of E16.5 wild-type, *Fgf18*$^{+/-}$, *Runx2*$^{+/-}$, *Fgf18*$^{+/-}$; *Runx2*$^{+/-}$, and *Fgf18*$^{-/-}$ embryos. (*D*) Real-time PCR analysis of α*1 Integrin* expression in humeri of E15.5 wild-type, *Runx2*$^{+/-}$, *Fgf18*$^{+/-}$, *Fgf18*$^{-/-}$, and *Runx2*$^{+/-}$; *Fgf18*$^{+/-}$ embryos, normalized to β-*actin*.

To verify molecularly the existence of an interaction between *Runx2* and *Fgf18*, we analyzed through real-time PCR expression of α*1 integrin*, a target gene of *Fgf18* expressed in proliferating chondrocytes (Supplementary Fig. 5; Davidson et al. 2005). Expression of α*1 integrin* was reduced 80% in *Fgf18*$^{-/-}$ compared with wild-type humeri (Fig. 3D). The same was true in humeri of heterozygous *Fgf18*$^{+/-}$; *Runx2*$^{+/-}$ embryos. In contrast, it was reduced only 40% in humeri of *Fgf18*$^{+/-}$ or *Runx2*$^{+/-}$ embryos (Fig. 3D). Taken together, these studies establish the existence of a genetic interaction between *Fgf18* and *Runx2* taking place in perichondrial cells and resulting in a delay in chondrocyte maturation.

Regulation of Fgf18 *expression by Runx2*

To establish molecularly that *Fgf18* is a transcriptional direct target of *Runx2*, we analyzed the *Fgf18* locus and found two potential Runx2-binding sites in the promoter region (sites 1 and 2) (Fig. 4A). The relevance of these sites was first tested by electric mobility shift assay (EMSA) using wild-type osteoblast nuclear extracts (NEs) and, as probes, labeled double-stranded oligonucleotides containing sequences covering them. A protein–DNA complex formed upon incubation of site 1 or of site 2 with osteoblast NEs (Fig. 4B). Moreover, an antibody against Runx2 supershifted these protein–DNA complexes, while antibodies against Sp1 did not. Second, to verify that Runx2 binds to the two sites present in the *Fgf18* promoter in vivo, we performed chromatin immunoprecipitation analyses using primary osteoblasts. Antibodies against Runx2 immunoprecipitated the regions of the *Fgf18* promoter containing either site 1 or site 2, while they could not immunoprecipitate *Fgf18* coding sequences.

To assess the importance of sites 1 and 2 in determining *Fgf18* promoter activity, we performed DNA transfection experiments in ROS17/2.8 osteoblastic cells that express *Runx2* (Ducy and Karsenty 1995). We used as a reporter construct a vector containing a 1.6-kb fragment of the *Fgf18* promoter fused to the luciferase gene (*pFgf18-Luc*) and as a positive control the 147-bp fragment of the *Osteocalcin* gene2 promoter fused to the luciferase gene (*pOG2-luc*). A mutation in either site 1 or site 2 of the *Fgf18* promoter caused a 40% decrease of *pFgf18-Luc* activity, while a mutation in both sites decreased it 60% (Fig. 4D). This result suggested that, in the conditions of this assay, the two Runx2-binding sites played an equally important role in determining activity of this fragment of *Fgf18* promoter in cells of the osteoblast lineage, although other transcription factors must regulate *Fgf18* promoter activity since mutation of these two sites decreased activity of this promoter only twofold. To further ascertain the importance of Runx2 as a regulator of *Fgf18* promoter activity, we next used COS cells that do not express *Runx2* or *Fgf18* to perform DNA cotransfection experiments. In this assay, Runx2 transactivated equally well *pFgf18-Luc* or *pOG2-luc*. Moreover, mutations in either site 1 or site 2 in the *Fgf18* promoter decreased Runx2 ability to transactivate *pFgf18-Luc* threefold, while mutations in both sites abolished it (Fig. 4E). Taken together, these data are consistent with the hypothesis that *Fgf18* is a transcriptional target of Runx2.

Various experimental evidence gathered mostly in chick has convincingly shown that the poorly differentiated mesenchymal cells making up the perichondrium influence negatively chondrocyte maturation (Long and Linsenmayer 1998; Di Nino et al. 2001). In an effort to provide a molecular basis for this function of perichondrium, we followed the unexpected observation that *Twist-1*, a gene expressed in perichondrial cells but not in chondrocytes, favors chondrocyte maturation. That this function of Twist-1 occurred through the Twist box along with *Runx2* expression in the perichondrium led us to show through several loss-of-function experiments that Runx2 regulates *Fgf18* expression in perichondrium and thereby inhibits chondrocyte maturation. Since *Runx2* expression is limited to part of the perichondrium, it is likely that other transcription factors may be involved in the regulation of chondrocyte maturation by the perichondrium. Likewise, we cannot rule out that Runx2 may have other targets in perichondrial cells that could explain the role of perichondrium on chondrocyte maturation.

Although no tools are available yet to delete genes in perichondrial cells only, several lines of evidence suggest that the *Twist-1–Runx2–Fgf18* cascade described here takes place primarily in the perichondrium. First, it was identified through the analysis of mutants in *Twist-1*, a gene that is neither expressed nor active in proliferating chondrocytes; second, *Twist-1* and *Runx2* that are both

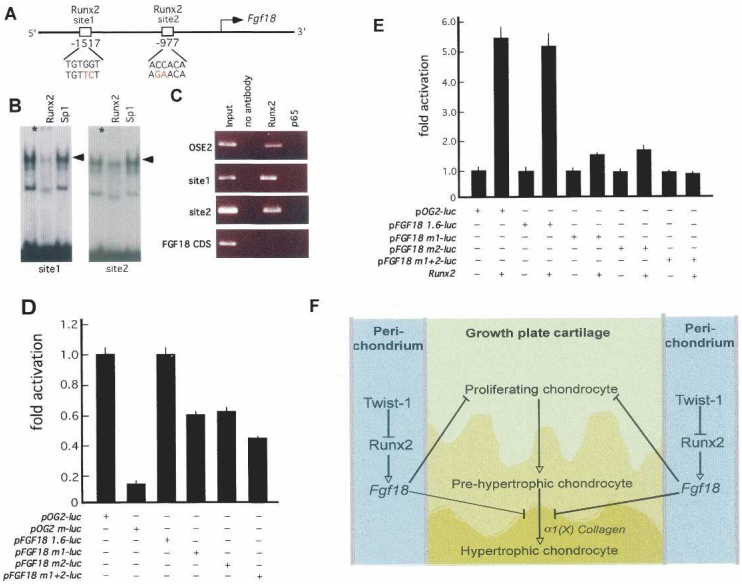

Figure 4. *Fgf18* expression is regulated by Runx2. (*A*) Schematic representation of putative Runx2-binding sites in the promoter of *Fgf18*. (*B*) EMSA. Osteoblast NEs were incubated with labeled oligonucleotides encompassing *Fgf18* sites 1 and 2. A protein–DNA complex formed upon incubation of NEs with all sites examined. Osteoblast NEs were incubated with antibodies against Runx2 or Sp1 prior to incubation with labeled oligonucleotides. Only antibody against Runx2 decreased binding to the all *Fgf18* site (1 and 2) oligonucleotides. (*C*) ChIP assay. An antibody against Runx2 immunoprecipitated site 1 and site 2 of the *Fgf18* promoter and the *Osteocalcin* promoter (OSE) but not *Fgf18* coding sequence (CDS). In another negative control experiment, an antibody against the P65 component of NF-κB did not immunoprecipitate these two sites. (*D*) ROS17/2.8 cells were transiently transfected with wild-type 1.6-kb *Fgf18* promoter-*luc* (p*Fgf18* 1.6-*luc*), *Fgf18* promoter-*luc*, or *pOG2-luc* constructs with either site 1 or site 2, or both sites were mutated (p*Fgf18* m1-*luc*, p*Fgf18* m2-*luc*, or p*Fgf18* m1 + 2-*luc*, respectively). (*E*) COS7 cells were transiently transfected with either p*Fgf18* 1.6-*luc*, p*Fgf18* m1-*luc*, p*Fgf18* m2-*luc*, p*Fgf18* m1 + 2-*luc*, or *pOG2-luc* constructs with or without *Runx2* expression vector. Runx2 increased activity of p*Fgf18* 1.6-*luc*, but not of p*Fgf18* m1 + 2-*luc*. (*F*) Proposed model. Twist-1 regulates Runx2 activity in perichondrial cells where *Fgf18* is a target gene of Runx2. As a result, through its perichondrial expression, Runx2 exerts a negative influence on chondrocyte maturation.

expressed in perichondrial cells interact in this process; third, *Fgf18* expression can only be detected in the perichondrium. We should emphasize that this genetic cascade may not be limited to the perichondrium, and it could also explain the craniosynostosis of *Twist*$^{+/-}$ mice since *Fgf18* is known to regulate suture closure in the skull (Liu et al. 2002; Ohbayashi et al. 2002). In addition, the absence of *osteocalcin* expression observed in *Runx2*$^{+/-}$; *Fgf18*$^{+/-}$ embryos suggests that *Runx2* may exert through *Fgf18* a negative influence on osteoblast differentiation; this would be consistent with the increase in osteoblast differentiation observed in *Fgf18*$^{-/-}$ embryos and implies that Runx2 exerts positive and negative influences on bone formation (Liu et al. 2002; Ohbayashi et al. 2002).

Based on this and previous studies, it appears that Runx2 has more complex functions during chondrogenesis than initially thought (Fig. 4F). First, early during skeletogenesis and through its expression in prehypertrophic chondrocytes, *Runx2* triggers chondrocyte hypertrophy in part by up-regulating α*1(X) Collagen* expression (Takeda et al. 2001; Ueta et al. 2001; Zheng et al. 2003). Subsequently, through its longer lasting perichondrial expression, *Runx2* exerts a negative influence on chondrocyte proliferation and hypertrophy. These antagonistic functions of Runx2 during chondrogenesis may help regulate bone formation. Although further experiments will be needed to test the validity of this model, results presented in this and previous studies illustrate the dynamic and complex influences that Runx2 exerts on multiple cell differentiation events during skeletogenesis. In that respect, Runx2 and the Sox proteins appear to be the main transcriptional architects of skeletogenesis.

Materials and methods

Mutant animals

CC/CC, *Runx2-*, *Runx3-*, and *Fgf18*-deficient mice, and α*1(I) Collagen-Cre* and α*1(II) Collagen-Cre* transgenic mice were described previously (Ducy et al. 1997; Dacquin et al. 2002; Li et al. 2002; Liu et al. 2002; Bialek et al. 2004). α*1(I) Collagen-Twist and N-Twist* transgenic mice were generated using standard procedures. Genotyping was performed by PCR analysis of genomic DNA.

In situ hybridization, immunohistochemistry, and BrdU incorporation assay

In situ hybridization was performed using ^{35}S-labeled riboprobes. Hybridizations were performed at 60°C. Autoradiography and Hoechst 33258 staining were performed as described (Takeda et al. 2001). HA-Twist-1 was detected with a mouse anti-HA antibody (1:1000; Roche) using the ABC Elite Kit (Vector Laboratories) and NovaRed (Vector Laboratories) as a substrate. For the proliferation assay, pregnant mice were injected with 500 µL of 10 mM BrdU 1 h before sacrifice. BrdU incorporation was detected using a Zymed BrdU staining kit. Sections were counterstained with hematoxylin. At least six embryos of each genotype were analyzed for each group and for each figure. Statistical significance was assessed by the Student's test.

RNA analysis and DNA transfection experiments

Total RNA was isolated from the indicated source, and real-time PCR was performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). COS and ROS 17/2.8 cells were grown in DMEM and DMEM-F-12, respectively, with 10% FBS. The 1.6-kb fragment of the *FGF18* promoter was PCR-amplified from genomic DNA, and its sequence was verified and cloned into p*GL3-luc*. Mutagenesis was performed using a mutagenesis kit (Stratagene) as per the manufacturer's instructions. Results are expressed as fold induction compared with single transfection of each pair. An asterisk indicate a *P* value <0.05; a double asterisk indicates a *P* value <0.01.

DNA-binding assay and chromatin immunoprecipitation (ChIP) assay

Labeled double-stranded oligonucleotides were prepared and EMSA was performed as described (Ducy and Karsenty 1995). Antibodies were added to the EMSA reaction and incubated on ice for 30 min prior to loading onto a 5% acrylamide gel. The ChIP assay was performed as per the manufacturer's instructions (Upstate Biotechnology) using primary osteoblasts. PCR primer sequences are available upon request. Primary osteoblasts were prepared and cultured as described (Ducy and Karsenty 1995).

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