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Molecular Pathogenesis of Genetic and Inherited Diseases

Down-Regulation of Ubiquitin Ligase Cbl Induced by Twist Haploinsufficiency in Saethre-Chotzen Syndrome Results in Increased PI3K/Akt Signaling and Osteoblast Proliferation

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Genetic mutations of Twist, a basic helix-loop-helix transcription factor, induce premature fusion of cranial sutures in Saethre-Chotzen syndrome (SCS). We report here a previously undescribed mechanism involved in the altered osteoblastogenesis in SCS. Cranial osteoblasts from an SCS patient with a Twist mutation causing basic helix-loop-helix deletion exhibited decreased expression of E3 ubiquitin ligase Cbl compared with wild-type osteoblasts. This was associated with decreased ubiquitin-mediated degradation of phosphatidyl inositol 3 kinase (PI3K) and increased PI3K expression and PI3K/Akt signaling. Increased PI3K immunoreactivity was also found in osteoblasts in histological sections of affected cranial sutures from SCS patients. Transfection with Twist or Cbl abolished the increased PI3K/Akt signaling in Twist mutant osteoblasts. Forced overexpression of Cbl did not correct the altered expression of osteoblast differentiation markers in Twist mutant cells. In contrast, pharmacological inhibition of PI3K/Akt, but not ERK signaling, corrected the increased cell growth in Twist mutant osteoblasts. The results show that Twist haploinsufficiency results in decreased Cbl-mediated PI3K degradation in osteoblasts, causing PI3K accumulation and activation of PI3K/Akt-dependent osteoblast growth. This provides genetic and biochemical evidence for a role for Cbl-mediated PI3K signaling in the altered osteoblast phenotype induced by Twist haploinsufficiency in SCS. ***(Am J Pathol 2006, 169:1303–1311; DOI: 10.2353/ajpath.2006.060102)***

Saethre-Chotzen syndrome (SCS), also called acrocephalosyndactyly III (ACS III), is an autosomal dominant hereditary disorder characterized clinically by facial dysmorphism, digit defects, and premature fusion of coronal sutures (craniosynostosis).[1–3] This disorder is induced by multiple genetic mutations in the gene for Twist, a basic helix-loop-helix (bHLH) factor involved in mesodermal differentiation. Most Twist mutations in SCS are located in the highly conserved bHLH domain.[4,5] Twist mutations in SCS cause Twist protein degradation, resulting in Twist haploinsufficiency, loss of dimerization with E proteins, and reduced binding to DNA canonical sequences in the promoter of target genes.[6,7] Despite the important implication of Twist mutations in craniosynostosis in SCS, our knowledge of the molecular mechanisms by which Twist alters the osteoblast phenotype in SCS remains incomplete. Our previous studies showed that Twist haploinsufficiency induced by deletion of the bHLH domain in SCS alters the osteoblast phenotype by affecting signaling molecules that control cell differentiation and apoptosis.[8–11] In addition, Twist was found to inhibit the functional activity of Runx2, a master gene controlling osteoblast differentiation in the developing mouse,[12] suggesting that multiple mechanisms may contribute to the altered osteoblast phenotype in SCS. However, the signaling pathways that act downstream of Twist and are involved in the altered osteoblast recruitment in SCS remain primarily unknown.

Proteasome degradation of ubiquitin-targeted proteins is an important mechanism that negatively controls activated signaling pathways.[13] Cbl is an E3 ubiquitin ligase that targets tyrosine kinase receptors and other signaling proteins, resulting in their ubiquitination and down-regulation.[14,15] In bone, Cbl regulates osteoclast activity by interacting with Src and associated proteins.[16] In osteoblasts, we previously showed that increased Cbl recruitment induced by fibroblast growth factor receptor-2 (FGFR2)-activating mutations in Apert syndrome results

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in increased ubiquitin-mediated degradation of FGFR2, Src proteins, and α5 integrin subunit, resulting in altered osteoblast differentiation and survival.[17,18] Among other proteins, Cbl proteins can interact with the p85-regulatory subunit of phosphatidyl inositol 3 kinase (PI3K), resulting in PI3K ubiquitylation and degradation.[19,20] PI3K catalyzes the production of phosphatidylinositol-3,4,5-trisphosphate and thereby contributes to the activation of various signaling components involved in the regulation of gene expression and cell survival.[21,22] In bone, PI3K controls osteoblast differentiation and survival[23] by interacting with local signaling factors[24–27] and Runx2.[28] In nonskeletal cells, PI3K was found to control cell growth through activation of the downstream Akt signaling pathway.[22,29–31] Consequently, deregulation of PI3K activity may lead to increased cell growth and tumor formation.[32–34] Nothing is known, however, of the role of PI3K in osteoblast growth and bone pathology.

In this study, we investigated the role of Cbl and PI3K in the abnormal osteoblast phenotype induced by Twist haploinsufficiency in SCS. We show here that Twist haploinsufficiency in human calvarial osteoblasts is associated with decreased Cbl expression resulting in PI3K accumulation, increased PI3K/Akt signaling, and osteoblast proliferation, a mechanism that may contribute to the premature cranial ossification in the SCS.

Materials and Methods

Bone Samples and Immunohistochemistry

Calvaria bone samples at the coronal suture level from three infants (age, 3.5 to 7 months) with SCS were obtained by surgical operation, and normal calvaria bone samples at equivalent areas were obtained from three normal age-matched infants who underwent local reconstruction of the skull unrelated to bone diseases, according to the French ethical committee recommendations.[10] The mutations studied cause Twist haploinsufficiency by inducing either degradation of truncated Twist protein (Y103X, Q109X) or loss of Twist DNA binding capacities (R118C).[6,7] Coronal sutures from patients and controls were fixed in 10% formaldehyde and embedded in paraffin, deparaffinized in xylene, and rehydrated through a graded series of ethanol. Sections were digested with 20 μg/ml proteinase K for 15 minutes at 37°C. Endogenous peroxidase was quenched with 0.3% H_2O_2 for 1 hour.[10] Sections were permeabilized with 0.1% Triton X-100, at 4°C for 2 minutes, incubated for 1 hour at 37°C with anti-PI3K (1/40; Cell Signaling, Danvers, MA), and the signal was revealed with diaminobenzidine. Negative controls were obtained by omitting the primary antibody from the reaction.

Cell Culture and Proliferation

Calvaria cell populations obtained by collagenase digestion from coronal sutures in one SCS subject with the Y103X mutation, which leads to deletion of the functional bHLH domain, and from an age-matched normal patient were immortalized and called mutant (M-Tw) and normal (wild-type) immortalized calvaria cell populations, respectively.[8] The osteoblast phenotype in these cells is consistent with the phenotype obtained in primary human calvaria cells *in vitro* and *in vivo*.[8,10] The cells were cultured in Dulbecco's modified Eagle's medium supplemented with glutamine (292 mg/L), 10% heat-inactivated fetal calf serum, and antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin). For analysis of cell growth, M-Tw and wild-type cells were plated at preconfluence and cultured for 72 hours in the presence of the PI3K inhibitor LY294002 (5 μmol/L; Calbiochem VWR, Fontenay, France), the Akt inhibitor SH-5, which does not decrease the phosphorylation of other kinases (20 μmol/L; Calbiochem), or the MAPK kinase 1 (MEK 1) inhibitor PD98059 (20 μmol/L; Calbiochem) or their solvent, and cell replication was measured using the 5-bromo-2′-deoxyuridine (BrdU) enzyme-linked immunosorbent assay (Roche, Neuilly sur Seine, France) according to the manufacturer's instructions. Data were expressed as absorbance in optical density and reported as the mean ± SEM of 8 to 10 replicates.

Transient Transfection Assays

To determine the implication of Twist and Cbl, Twist mutant cells were plated at 2500 cells/cm^2 the day before transfection. The cells were co-transfected with the plasmid (2.5 μg/3 cm^2 dish) and pSV-β-galactosidase (50 ng of β-gal) control vector (Promega, Charbonnières, France) in Dulbecco's modified Eagle's medium with 1% fetal calf serum. Cells were incubated for 48 hours with empty expression vector (pBK or pcDNA3), pBK-Cbl, or pcDNA3-Twist vector and Exgen (Euromedex, Souffelweyersheim, France) according to the manufacturer's directions. Efficiency of transfection was ~15 to 20% at 48 hours after transfection as controlled by β-gal activity (β-gal reporter gene assay; Roche). Although the transfection efficiency was low in these cells, the strong promoter (CMV) used in plasmids allowed a high level of expression of exogenous Twist or Cbl in the cell population.

Proteasome Activation

To determine whether PI3K levels may be rescued by activation of the proteasome, permeabilized Twist mutant cells were treated with the PA28 proteasome activator (200 μmol/L; Calbiochem). After 48 hours, phosphorylated PI3K (p85) protein levels were determined by Western blot analysis as described below.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) Analysis

The mRNA levels of Cbl, PI3K (p85), and the osteoblast marker genes Runx2, type I collagen (COLIA1), and osteocalcin were analyzed by RT-PCR.[11] Cells were washed with phosphate-buffered saline and lysed with Extract-All (Eurobio, Courtaboeuf, France) reagent according to the manufacturer's instructions. Three μg of

total cellular RNA from each sample were reverse-transcribed, and the cDNA samples were then divided and amplified by PCR using specific primers. Primers for PI3K (p85) were for sense 5′-AGCACCGACTTCAAGACTACG-3′ and for anti-sense 5′-GGATGCCAATGAGATTGTCC-3′. Primers for Cbl, Runx2, COLIA1, and GAPDH were as described.[11] Autoradiographic signals were quantified using a scanner densitometer, and the signal for each gene was related to that of GAPDH.

Western Blot and Immunoprecipitation Analyses

Cell proteins were extracted in RIPA buffer with 1 mmol/L phenylmethyl sulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 nmol/L calyculin A, 50 nmol/L microcystin LR, 2 mmol/L Na_3VO_4.[35] Lysates were clarified by centrifugation at 12,000 $\times$ *g* for 30 minutes at 4°C, and protein content of the supernatants was determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). For Western blot analysis, equal aliquots (50 μg of protein lysates were electrophoresed in 4 to 20% sodium dodecyl sulfate-polyacrylamide gradient gels. Electrophoresed proteins were transferred onto polyvinylidene difluoride membranes (Hybond-P; Amersham, Saclay, France). The membranes were then reacted with antibodies for phospho-PI3K (Cell Signaling), total PI3K (1/1000; Cell Signaling), phospho-Akt (1/500; Cell Signaling), total Akt (1/500, Cell Signaling) that recognizes the three Akt forms, or β-actin (1/200; Sigma, St. Louis, MO), incubated for 1 hour with the appropriate affinity-purified anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA), probed with peroxidase-coupled specific secondary antibodies and visualized using the enhanced chemiluminescence detection kit (Amersham). The levels of proteins were measured by scanning densitometry and corrected for actin.

For immunoprecipitation analysis, equal aliquots (100 μg) of protein lysates were immunoprecipitated using 2.5 μg of specific anti-Cbl (Santa Cruz) or anti-PI3K (Cell Signaling) and incubated overnight at 4°C in a rotating device. After 24 hours, 20 μl of protein A/G agarose (Santa Cruz) were added and incubated for 1 hour at 4°C. Immunoprecipitates were then collected by centrifugation at 1200 $\times$ *g* for 3 minutes, and the pellets were washed four times with lysis buffer and resuspended in 25 μl of running buffer. Aliquots were then subjected to electrophoresis as described above, and membranes were reacted with PI3K, phospho-PI3K (Cell Signaling), or ubiquitin (Cell Signaling) antibodies. Immunoblots were probed with peroxidase-coupled specific secondary antibodies and visualized by enhanced chemiluminescence.

Data Analysis

The data are representative of two to four different experiments. Differences between the mean values $\pm$ SEM were analyzed using the statistical package superanalysis of variance (Macintosh; Abacus Concepts, Inc., Berkeley, CA) with a minimal significance of $P < 0.05$.

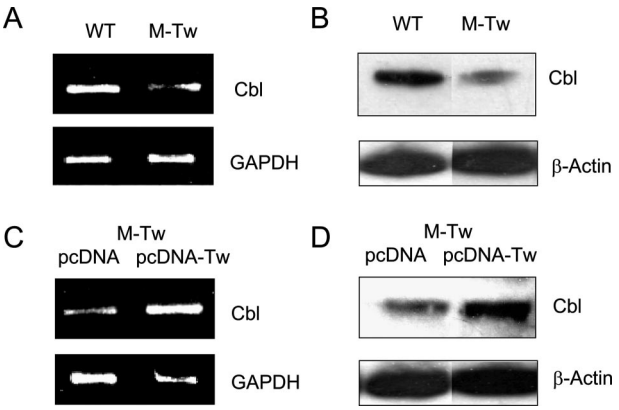

Figure 1. Twist haploinsufficiency reduces Cbl expression in cranial osteoblasts. **A:** Total RNA from calvaria osteoblasts from a coronal suture in an SCS patient with Y103X Twist mutation (M-Tw) and wild-type (WT) human calvaria osteoblasts was subjected to RT-PCR analysis to detect Cbl levels, and GAPDH was used as internal control. **B:** Western blot analysis showing decreased Cbl protein levels in M-Tw cells compared with WT cells. **C** and **D:** M-Tw cells were transfected with a Twist expression vector or control vector (pcDNA3), and Cbl mRNA levels were determined. Transient transfection with Twist increased Cbl mRNA levels compared with cells transfected with the empty vector. **D:** Western blot analysis confirmed the increased Cbl protein levels in Twist-transfected M-Tw cells compared with cells transfected with the empty vector.

Results

Twist Haploinsufficiency Reduces Cbl Expression in Osteoblasts

To analyze the potential role of Cbl in osteoblast pathology in SCS, Cbl expression was determined in wild-type osteoblasts and Twist mutant osteoblasts expressing the natural Twist Y103X mutation causing bHLH deletion and Twist haploinsufficiency.[6–8] RT-PCR analysis showed that Twist mutant cells expressed decreased Cbl mRNA levels compared with wild-type cells (Figure 1A). Decreased Cbl levels at the protein level were also found in Twist mutant osteoblasts compared with wild-type osteoblasts, as demonstrated by Western blot analysis (Figure 1B). To further determine the role of Twist in the alteration of Cbl expression, mutant osteoblasts were transfected with Twist expression vector, and Cbl protein levels were determined. Western blot analysis showed that forced expression of Twist in mutant osteoblasts corrected Cbl expression at both the RNA and protein levels (Figure 1, C and D). These results show that Twist haploinsufficiency is associated with decreased Cbl expression in Twist mutant osteoblasts, suggesting that Twist may control Cbl at the transcriptional and/or posttranscriptional level.

Increased PI3K Levels and Signaling in Twist Mutant Osteoblasts

Cbl is an adaptor protein that interacts with PI3K and mediates its ubiquitination and proteasome degradation.[14,15] We therefore hypothesized that Cbl may interact physically with PI3K in osteoblasts to mediate ubiquitination of this protein. As shown in Figure 2A, Cbl was found to co-immunoprecipitate with PI3K (p85) in Twist mutant osteoblasts and wild-type cells, indicating physical inter-

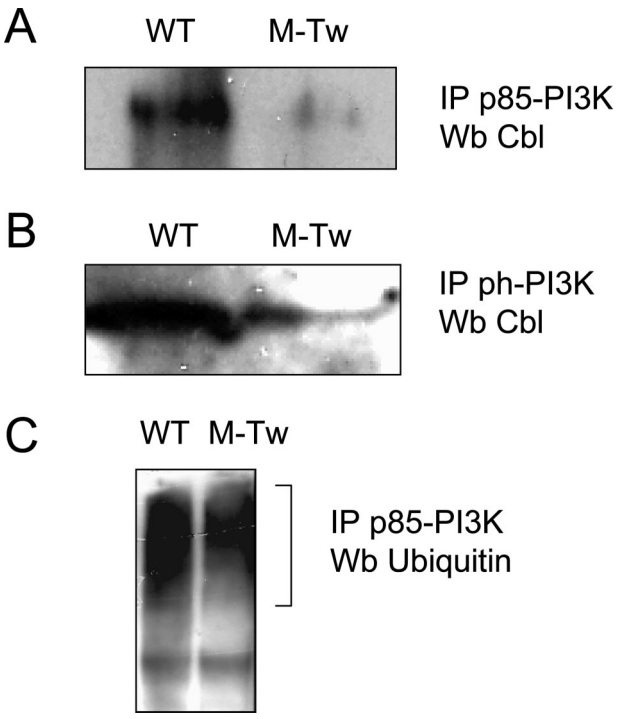

Figure 2. Cbl interacts with PI3K and ubiquitin in Twist mutant osteoblasts. Cell lysates from wild-type (WT) and Twist mutant (M-Tw) osteoblasts were immunoprecipitated (IP) with total p85-PI3K antibody or phosphorylated (ph)-PI3K, resolved with sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and blotted with anti-Cbl (**A**, **B**) or ubiquitin (**C**) antibodies.

actions between the two proteins. The levels of total and phosphorylated PI3K associated with Cbl were much lower in Twist mutant cells compared with wild-type cells (Figure 2, A and B) as a result of the decreased amount of Cbl protein in mutant osteoblasts (Figure 1A). Further immunoprecipitation studies showed decreased PI3K polyubiquitination in mutant osteoblasts compared with control cells (Figure 2C). Overall, these results show that the decreased Cbl level in Twist mutant cells is associated with decreased PI3K protein ubiquitination and degradation.

To assess whether this effect results in alteration of PI3K levels and signaling, we determined total and phosphorylated PI3K levels in Twist mutant osteoblasts. As shown in Figure 3A, Western blot analysis revealed a significant increase in total PI3K (p85) protein level in Twist mutant cells compared with wild-type cells. In addition, the amount of phosphorylated PI3K (p85) was increased in Twist mutant osteoblasts compared with wild-type cells (Figure 3A). In contrast, RT-PCR analysis showed a similar amount of p85-PI3K transcripts in Twist mutant cells compared with wild-type cells (Figure 3B). Because Akt/PKB acts as a downstream effector of PI3K, we analyzed the alteration of this signaling pathway in Twist mutant osteoblasts. As shown in Figure 3C, phosphorylated Akt levels were increased in Twist mutant cells compared with wild-type osteoblasts. These results indicate that PI3K accumulation induced by Twist haploinsufficiency is associated with increased PI3K/Akt signaling in mutant osteoblasts.

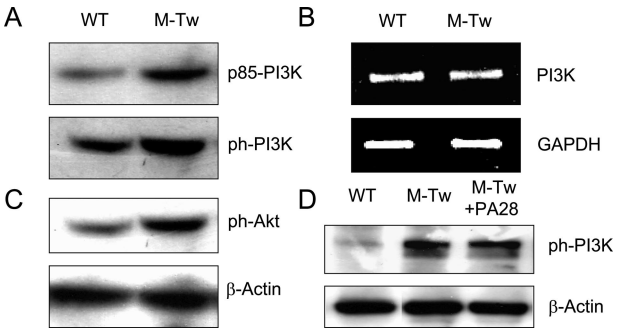

Figure 3. Twist mutant osteoblasts show increased PI3K/Akt signaling *in vitro*. **A** and **C:** Twist mutant (M-Tw) and wild-type (WT) osteoblasts were lysed, and equivalent amounts of proteins were analyzed for total PI3K, phosphorylated (ph)-PI3K, or ph-Akt by Western blotting. **B:** mRNA levels for PI3K (p85) were analyzed in M-Tw and WT osteoblasts by RT-PCR analysis. **D:** Permeabilized M-Tw osteoblasts were treated with the PA28 proteasome activator (200 μmol/L) or the solvent for 48 hours, and ph-PI3K level was determined by Western blotting. β-Actin was used as internal control of protein loading.

The increased PI3K protein levels may arise from change in translational efficiency as well as increased protein stability. Because Cbl interacts with PI3K to control its stability, we investigated whether the increased PI3K signaling in mutant cells may result from reduced proteasome degradation. Permeabilized Twist mutant cells were treated with the PA28 proteasome activator and phosphorylated PI3K levels were determined by Western blot analysis. Activation of proteasome with PA28 slightly decreased phosphorylated PI3K levels in Twist mutant cells (Figure 2D). The effect observed, however, was not striking most likely because of the low penetration within cells of PA28 resulting from the large size of the proteasome activator. However, collectively the results suggest that the increased PI3K protein levels and activity in Twist mutant osteoblasts results, at least in part, from decreased degradation by the proteasome.

Increased PI3K Expression in Human Cranial Sutures in SCS

Because we used human osteoblasts directly derived from affected coronal tissues, the observed *in vitro* phenotype is likely to reflect the *in vivo* situation in SCS. To confirm that the increased PI3K expression *in vitro* also occurs *in vivo*, we examined PI3K expression in histological sections of coronal sutures in a 3-month-old SCS patient with the Y103X mutation compared with age-matched controls. The immunohistochemical analysis of coronal sutures in this SCS patient showed increased PI3K expression in osteoblasts and mesenchymal cells compared with normal sutures from age-matched controls (Figure 4). Similar findings were observed in two patients with Twist nonsense mutation (Q109X) or missense mutation (R118C) compared with normal sutures from age-matched controls (Figure 4). These results indicate that Twist haploinsufficiency in SCS is associated with increased PI3K levels in osteoblasts in fused cranial sutures *in vivo*, confirming the *in vitro* analysis in calvaria osteoblasts.

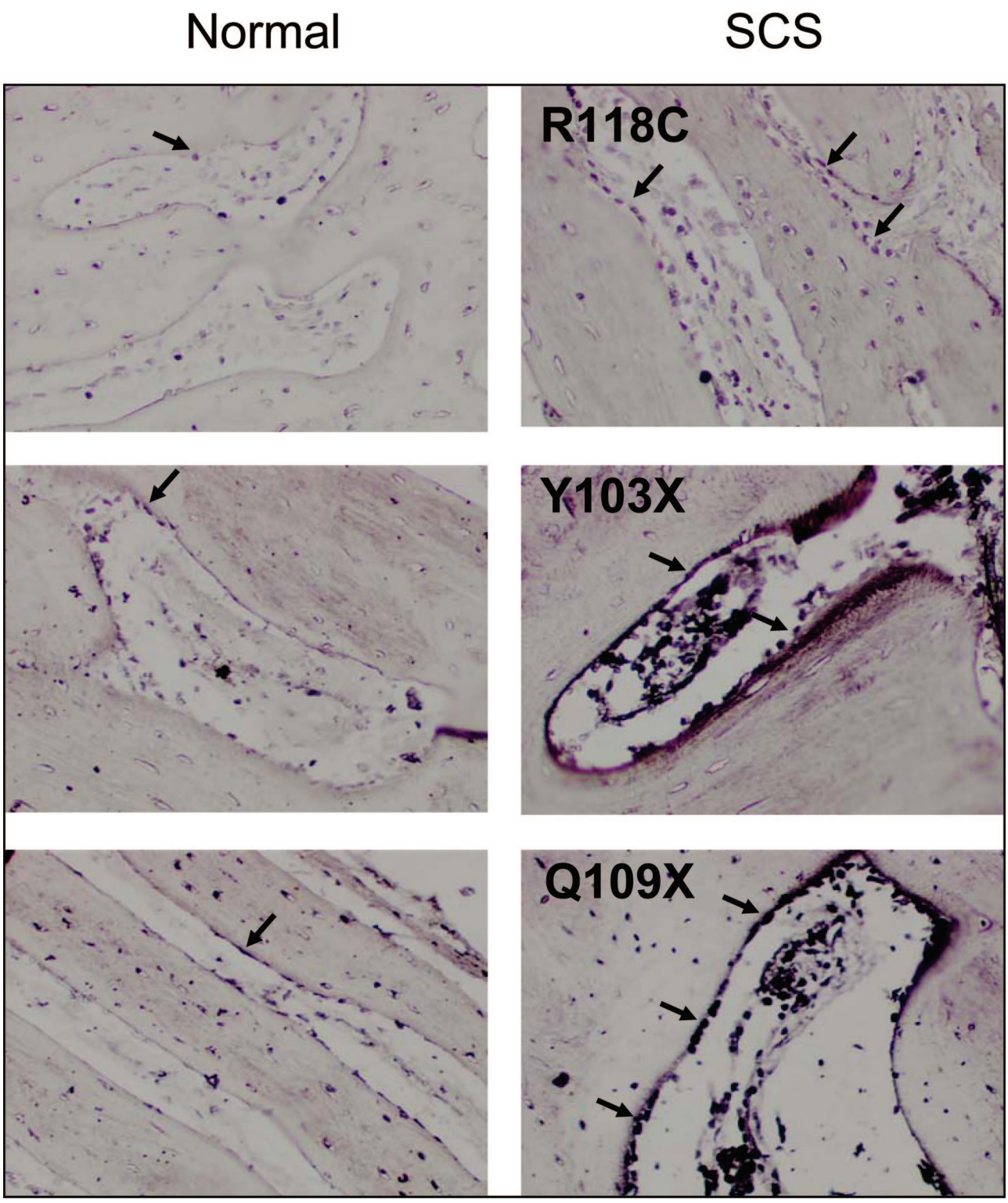

Figure 4. Immunohistochemical analysis of PI3K in human coronal sutures in patients with SCS. Coronal sutures in SCS patients with Twist mutations (Y103X, Q109X, R118C) inducing Twist haploinsufficiency showed increased total PI3K (p85) immunoreactivity in osteoblasts (**arrows**) compared with normal sutures from age-matched controls. Original magnifications, ×125.

Forced Expression of Twist Restores PI3K/Akt Signaling in Twist Mutant Osteoblasts

To assess the role of Twist in the observed alteration of Cbl and PI3K expression in SCS, Twist mutant cells were transfected with Twist expression vector, and changes in Cbl, total PI3K, and phosphorylated p85-PI3K levels were determined. As shown in Figure 5A, total p85-PI3K levels were increased in Twist mutant osteoblasts compared with wild-type cells, confirming our previous analysis.

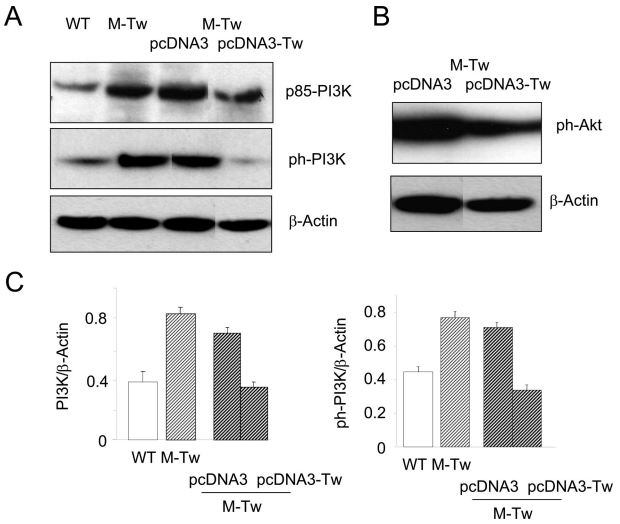

Figure 5. Forced expression of Twist restores PI3K/Akt signaling in Twist mutant osteoblasts. Twist mutant (M-Tw) osteoblasts were transfected with a Twist expression vector or control vector (pcDNA3) for 48 hours, and cell lysates were subjected to Western blot to detect total and phosphorylated (ph)-PI3K (**A**) or ph-Akt (**B**), and the data were compared with the levels in wild-type (WT) osteoblasts. **C:** The levels of PI3K and ph-PI3K were measured by scanning densitometry and corrected for β-actin, which was used as internal control of protein loading. The data are the mean ± SEM of four different experiments.

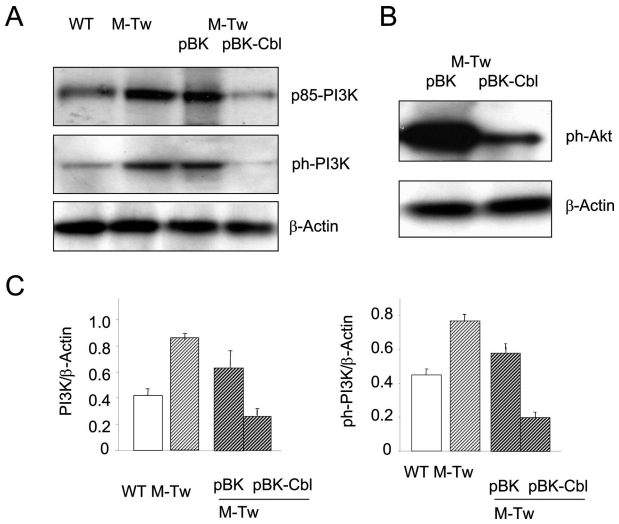

Figure 6. Forced expression of Cbl restores PI3K/Akt signaling in Twist mutant osteoblasts. M-Tw calvaria osteoblasts were transfected with Cbl expression vector or control vector (pBK) for 48 hours and cell lysates were subjected to Western blot to detect total and phosphorylated (ph)-PI3K (**A**) or ph-Akt (**B**), and the data were compared with the levels in wild-type (WT) osteoblasts. **C:** The levels of PI3K and ph-PI3K were measured by scanning densitometry and corrected for β-actin. The data are the mean ± SEM of five (PI3K) or three (ph-PI3K) different experiments.

Strikingly, forced expression of Twist in mutant osteoblasts decreased total PI3K protein as well as phosphorylated PI3K to the level in control cells (Figure 5A). Similar data were found in four different experiments (Figure 5C). Consistently, forced expression of Twist decreased phosphorylated Akt levels in Twist mutant cells (Figure 5B). These results suggest that the observed increase in PI3K/Akt signaling results from Twist haploinsufficiency in mutant osteoblasts.

Forced Expression of Cbl Restores PI3K/Akt Signaling in Twist Mutant Osteoblasts

To further determine the role of Cbl in the alteration of PI3K/Akt signaling in Twist mutant osteoblasts, mutant cells were transfected with Cbl, and PI3K levels were determined. Transfection with the Cbl expression vector rescued Cbl protein levels in Twist mutant osteoblasts (data not shown). Cbl transfection in Twist mutant osteoblasts led to decreases total PI3K expression to the level in control cells (Figure 6A). Similar results were found in five different experiments (Figure 6C). Consistently, Cbl overexpression corrected phosphorylated PI3K levels (Figure 6A), which was confirmed in five different experiments (Figure 6C). In addition, Cbl overexpression reduced phosphorylated Akt levels (Figure 6B). The finding that the rescue of Cbl restored PI3K/Akt signaling in Twist mutant osteoblasts indicates that the decreased Cbl expression protects PI3K from degradation, resulting in increased PI3K levels and increased PI3K/Akt signaling.

Lack of Role of PI3K in the Altered Osteoblast Differentiation in SCS

To assess the functional implication of Cbl-mediated alteration of PI3K degradation in the altered osteoblast phenotype induced by Twist haploinsufficiency, Twist mutant cells were transfected with Cbl expression vector, and osteoblast phenotypic markers were determined. As reported previously,[9,11] Runx2 mRNA levels were decreased in Twist mutant osteoblasts compared with wild-type cells (Figure 7A). Moreover, basal COLIA1 mRNA levels were increased in Twist mutant osteoblasts, whereas osteocalcin levels were decreased in Twist mutant osteoblasts compared with wild-type cells (Figure

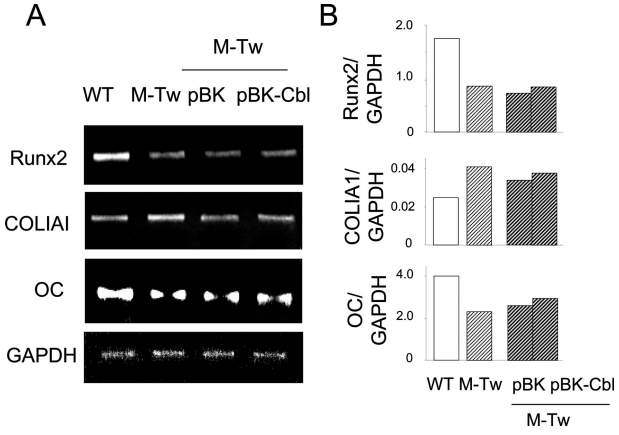

Figure 7. Lack of role of the increased PI3K signaling in the altered osteoblast differentiation markers in Twist mutant osteoblasts. Twist mutant (M-Tw) osteoblasts were transfected with Cbl expression vector or control vector (pBK). **A:** Total cellular RNA was subjected to RT-PCR analysis to detect the osteoblast markers Runx2, type I collagen (COLIA1), and osteocalcin (OC). **B:** Densitometric analysis of mRNA visualized by autoradiography was determined, and the level of expression was normalized to GAPDH.

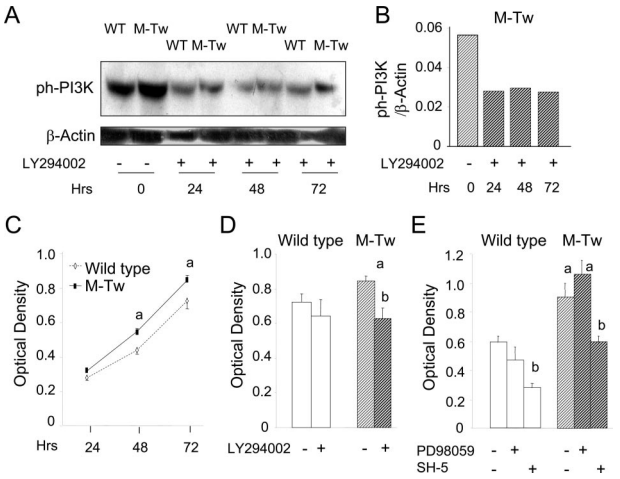

Figure 8. Role of PI3K in the increased osteoblast proliferation in Twist mutant osteoblasts. **A:** Twist mutant (M-Tw) and wild-type (WT) osteoblasts were treated with the PI3K inhibitor LY294002 (5 μmol/L, 24 to 72 hours) or its solvent, and cell lysates were subjected to Western blot analysis to detect phosphorylated (ph)-PI3K. **B:** Densitometric analysis was performed and the level of expression was normalized to β-actin. **C:** In parallel experiments, cell proliferation of M-Tw and WT cells was determined by the BrdU assay. **D** and **E:** Cell proliferation in M-Tw and WT cells was assessed at 72 hours in the presence of the PI3K inhibitor (LY294002, 5 μmol/L), MEK 1 inhibitor (PD98059, 20 μmol/L), Akt inhibitor (SH-5, 20 μmol/L), or their solvent. Data are the mean ± SEM of 10 values. [a]$P < 0.05$ versus WT cells; [b]$P < 0.05$ versus untreated M-Tw cells.

7A), confirming our previous findings.[9,11] We previously showed that ectopic expression with Twist expression vector restored normal osteoblast gene expression in Twist mutant cells.[11] In contrast, transfection with Cbl in Twist mutant osteoblasts did not correct Runx2 or osteocalcin mRNA levels, which remained lower than normal. Consistently, overexpression of Cbl did not correct COLIA1 mRNA levels in Twist mutant osteoblasts, which remained higher than in wild-type cells (Figure 7, A and B). Additional biochemical and histochemical analyses showed that alkaline phosphatase activity, an early marker of osteoblast function that was increased in M-Tw osteoblasts,[8] remained unaffected by Cbl overexpression in Twist mutant osteoblasts (data not shown). These findings suggest that the increased PI3K signaling is not implicated in the altered expression of osteoblast marker genes in Twist mutant osteoblasts.

Role of PI3K/Akt Signaling in the Increased Osteoblast Proliferation in SCS

Given the role of PI3K/Akt in the control of cell growth,[29–31] we then hypothesized that the increased PI3K signaling in Twist mutant osteoblasts may result in increased osteoblast cell proliferation. To test this hypothesis, Twist mutant cells and control cells were treated with the specific PI3K inhibitor LY294002, and cell proliferation was determined. As shown in Figure 8A, treatment with LY294002 decreased the levels of phosphorylated PI3K in wild-type and Twist mutant osteoblasts. The PI3K inhibitor LY294002 decreased the levels of phosphorylated PI3K by ~50% in M-Tw cells (Figure 8B), showing its efficient inhibitory effect on PI3K activity. The analysis of cell growth in basal conditions showed that Twist mutant cells exhibited greater than normal DNA replication rate at 48 and 72 hours of culture, as measured by the BrdU assay (Figure 8C). Strikingly, treatment with LY294002 significantly reduced cell proliferation in Twist mutant cells (Figure 8D). Similarly, selective pharmacological inhibition of Akt activity with the Akt inhibitor SH-5 corrected cell growth in Twist mutant cells (Figure 8E). In contrast, the MEK 1 inhibitor PD98059 had no significant effect on cell growth in Twist mutant cells (Figure 8E). Overall, these findings suggest that increased PI3K/Akt signaling mediates the increased cell proliferation in Twist mutant osteoblasts.

Discussion

The molecular mechanisms by which Twist haploinsufficiency results in osteoblast pathology and craniosynostosis in SCS are not fully understood. The present study indicates that Twist haploinsufficiency in SCS is associated with decreased expression of the E3 ubiquitin ligase Cbl, resulting in PI3K accumulation, increased PI3K/Akt signaling and osteoblast replication. We first showed that Twist haploinsufficiency resulted in increased Cbl expression in mutant osteoblasts, a feature that was rescued by Twist overexpression, suggesting that Twist regulates Cbl expression. Although the mechanism by which Twist may regulate Cbl expression remains unknown, it is tempting to assume that Cbl may be a direct target of Twist or Runx2, expression of which is altered in Twist mutant human osteoblasts.[9,11] It is, however, unknown whether functional Twist or Runx2 DNA binding sequences are present in the human Cbl promoter, which remains to be cloned. Alternatively, Twist haploinsufficiency may regulate Cbl expression by indirect mechanisms.[36] Regardless of the mechanism, our data indicate that the decreased Cbl expression in Twist mutant osteoblasts is associated with increased PI3K levels. These *in vitro* data appear to be relevant to the *in vivo* situation because PI3K expression was also increased in affected coronal sutures from patients with SCS. This reveals that Twist haploinsufficiency in SCS is associated with altered PI3K signaling in the osteoblast lineage *in vitro* and *in vivo*.

Our results point to one mechanism by which PI3K signaling is up-regulated in Twist mutant osteoblasts. Previous studies suggested that Cbl proteins may interact with PI3K and direct PI3K ubiquitination and proteasome-mediated degradation.[19,20] The reduced Cbl expression and the decreased PI3K associated with Cbl in Twist mutant osteoblasts suggest that the increased PI3K signaling in Twist mutant osteoblasts results, at least in part, from reduced Cbl-mediated PI3K degradation. Our finding that abnormalities in Cbl and PI3K levels can be rescued by ectopic Twist or Cbl suggests a role for Twist in the reduced Cbl-dependent PI3K degradation and subsequent increased PI3K signaling in Twist mutant osteoblasts. Recent studies indicate that PI3K activity is regulated by major regulatory molecules in osteoblastic cells. We previously showed that FGF2 modulates PI3K activity in human cranial osteoblasts.[24] Other studies indicate that bone morphogenetic

protein-2 and Runx2 interact with PI3K/Akt signaling in preosteoblastic cells.[25,28,37] The present finding indicates that PI3K signaling can also be regulated by ubiquitin-mediated degradation in osteoblasts. Consistently, recent studies point to a role of ubiquitination-mediated degradation processes in the control of osteoblast function and pathology. Notably, the activity of ATF4, which is involved in osteoblast-specific gene expression and in the pathophysiology of Coffin-Lowry syndrome,[38] is regulated by ubiquitination mediated by β-TRCP1, an E3 ubiquitin ligase interacting with ATF4.[39] Runx2, an essential transcription factor involved in osteoblast differentiation, is targeted to proteasomal degradation by interacting with the E3 ubiquitin ligase Smad regulatory factor (Smurf1).[40,41] In addition, Smurf1 negatively regulates osteoblast activity by controlling MEKK2 degradation and downstream JNK signaling.[42] A recent study indicates that Twist itself can be degraded by the proteasome.[43] Overall, this supports an important role of ubiquitin-mediated degradation in the control of osteoblastogenesis. Interestingly, we recently showed that Cbl interacts with FGFR2 and Src proteins to increase osteoblast differentiation in Apert syndrome.[17] Moreover, we found that the altered Cbl-mediated ubiquitination of α5 integrin subunit contributes to the increased osteoblast apoptosis induced by FGFR2 activation in Apert syndrome.[18] Thus, alteration of Cbl-mediated degradation of signaling proteins appears to be an important mechanism that contributes to the abnormal osteoblast phenotype in syndromic craniosynostosis.

One important question was to determine whether the up-regulation of PI3K signaling in Twist mutant osteoblasts may contribute functionally to the altered osteoblast phenotype in SCS. Osteoblast disorders in syndromic craniosynostosis may result from alterations in cranial cell proliferation, differentiation, and/or survival.[44] Increased PI3K/Akt signaling is known to mediate cell survival in nonskeletal cells[45] as well as skeletal cells.[24,27] However, a role of PI3K/Akt in osteoblast survival in SCS is unlikely because we found increased apoptosis in Twist mutant osteoblasts,[10] consistent with the anti-apoptotic role of Twist in nonskeletal cells.[35,46] Because PI3K may play a role in the control of osteoblast differentiation,[25–28] we sought to determine the implication of PI3K signaling in the altered expression of differentiation marker genes in SCS. The finding that Cbl transfection corrected PI3K activity but not osteoblast marker gene expression suggests that the increased PI3K signaling is not involved in the altered osteoblast differentiation in Twist mutant osteoblasts. This is also supported by our finding that the PI3K inhibitor LY294002 did not correct Runx2, COLIA1, or osteocalcin mRNA levels in Twist mutant osteoblasts (data not shown). Because cell replication is in part controlled by PI3K/Akt in nonskeletal cells,[29–31] a remaining possibility was that activation of PI3K/Akt may activate cell growth in Twist mutant osteoblasts. Strikingly, the findings that cell proliferation was increased in Twist mutant osteoblasts compared with wild-type cells and that pharmacological inhibition of PI3K or Akt signaling, but not MEK1 signaling, restored normal cell proliferation in mutant cells imply a selective role for PI3K/Akt signaling in the increased cell growth in Twist mutant cells. These results do not exclude other mechanisms from contributing to cell replication in Twist mutant osteoblasts. For example, Runx2 was recently shown to be a cell growth inhibitor in osteoblastic cells.[47–50] Whether the observed down-regulation of Runx2 in Twist mutant human osteoblasts[11] may contribute to the increased cell growth in these cells warrants further investigation.

In conclusion, the present data support a model by which Cbl down-regulation in Twist mutant osteoblasts induces PI3K accumulation, increased PI3K/Akt signaling, and cell proliferation. It can thus be hypothesized that the increased osteoblast replication induced by PI3K/Akt signaling may result in an increased number of bone-forming cells, which may contribute to the premature cranial suture ossification in SCS. These results reveal a novel Cbl-mediated mechanism controlling osteoblastogenesis and provide additional insights into the molecular mechanisms by which Twist haploinsufficiency alters the osteoblast phenotype in SCS.

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