The Boston-type Craniosynostosis Mutation MSX2 (P148H) Results in Enhanced Susceptibility of MSX2 to Ubiquitin-dependent Degradation*

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Boston-type craniosynostosis is caused by a single amino acid substitution, P148H, in the transcription factor MSX2. The increased binding affinity of MSX2 (P148H) to the response element has led many to hypothesize that the substitution is a gain-of-function mutation. However, there have been conflicting reports on the function of MSX2, and by extension, the nature of the P148H mutation remains unclear. In this study, we have examined the molecular mechanism of MSX2 function and the nature of the P148H mutation. During cranial suture closure of rodent, Msx2 expression was detected in the suture space. Over-expression of wild type MSX2 in mesenchymal cells stimulated cell proliferation and cyclin D1 expression, whereas P148H mutant did not. These results indicated that MSX2 is involved in maintaining the suture space by stimulating suture mesenchymal cell proliferation and that P148H is defective in this process. The protein levels of P148H were lower than wild type Msx2 (Msx2-WT), and pulse-chase experiments indicated that the mutant protein has a shorter half-life than the Msx2-WT protein. The ubiquitylation level of P148H was greater than that of Msx2-WT. The degradation of Msx2 was mediated by Praja1, and the P148H mutant was degraded more effectively than WT. The ubiquitylation of Msx2-WT was higher in the presence of Msx2 (P148H), which indicated that P148H functions as a dominant-negative mutant. Collectively, the primary function of MSX2 in suture closure is the induction of cell proliferation and suture maintenance, and the mutation results in an increased susceptibility of both wild type and mutant MSX2 to proteasomal degradation.

Msx2 was originally identified as a homolog of the Drosophila muscle segment homeobox gene msh and is one of the major molecular switch proteins in osteoblast differentiation (1). The expression of Msx2 is specifically induced during vertebrate embryonic development in regions of epithelial-mesenchymal interaction (2, 3). Genetic and developmental studies of Msx2 have shown that it plays a pivotal role in craniofacial (4) and limb development (3–6) and tissue organogenesis (7, 8).

There have been controversial reports in the literature on the molecular mechanism of Msx2 function in craniofacial bone formation. Several studies have shown that Msx2 functions as a transcriptional repressor of bone marker genes through interactions with other homeodomain proteins, as well as components of the core transcription complex (9–15). Msx2 is required for osteoblast differentiation and has been shown to suppress the gene promoters of the major bone maker genes alkaline phosphatase, osteocalcin, and Runx2-II in cell culture systems (9, 10, 13–15). In addition to the transcriptional repression of bone marker genes, Msx2 also inhibits the function of key osteogenic regulators, such as Runx2, Dlx3, and Dlx5, through the DNA binding-independent protein-protein interactions and/or the binding competition on the same DNA sequences (9, 11, 14). Moreover, Msx2 expression is higher in periodontal ligament and tendon cells than in osteoblastic cells (16), which suggests that Msx2 functions in a molecular defense capacity to prevent the ossification of the periodontal space. These results implicate Msx2 in the negative regulation of osteogenesis. On the other hand, studies have implicated Msx2 as a positive regulator of osteogenesis. Transient transduction studies using a retroviral expression system have shown that Msx2 suppresses the adiogenic differentiation of aortic myofibroblast cells through the inhibition of peroxisome proliferator-activated receptor γ (17). In addition, Msx2 has been shown to promote cardiovascular calcification through the activation of paracrine Wnt signal (18, 19). These results implicate Msx2 in the promotion of osteogenic differentiation.

Molecular genetic studies of Msx2 have shown that it is essential for normal suture closure and skull mineralization. Msx2 knock-out mice exhibit multiple abnormalities, such as persistent calvarial foramen, defective endochondral bone formation, and defective organogenesis in tooth, hair follicle, and mammary gland (8). Partial deletion mutations of MSX2 cause parietal foramina in humans (20, 21). Msx2 transgenic mice exhibit craniosynostosis with increased pools of proliferative osteogenic cells in the calvaria (22). Some MSX2 trisomy patients also exhibit craniosynostosis, which indicates that the
function of Msx2 is conserved in human (20, 21). Studies of Msx2 transgenic mice, which exhibit suture overgrowth and overlap without suture, have indicated that the mechanism of premature suture closure in these mice is due to a specific incremental increase in osteogenic cell proliferation (22, 23). These results provide evidence that Msx2 is a major regulator of the proliferation of Msx2-expressing osteoprogenitor cells and subsequent osteoblast differentiation.

Boston-type craniosynostosis is an autosomal dominant disorder that results in the premature fusion of calvarial bones, which leads to defects in skull and brain development (24, 25). The underlying mutation in Boston-type craniosynostosis is a single amino acid substitution in MSX2, P148H (26). Bacterially expressed recombinant Msx2 (P148H) exhibits a higher affinity than Msx2-WT\(^2\) for synthetic oligonucleotides that contain a core homeodomain response element (27). Based on the increased binding affinity of the mutant protein to DNA and the autosomal dominant transmission pattern of the disease, it has been hypothesized that MSX2 (P148H) is a gain-of-function mutation. However, because of the conflicting reports of MSX2 function, we have re-examined the expression and activity of MSX2 in osteoblast differentiation, cranial suture closure, or cell proliferation, we have re-examined the function of MSX2 and the nature of the MSX2 mutation in Boston-type craniosynostosis.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—C2C12, NSC-F5, 293T, SaOS-2, and ROS17/2.8 cells were maintained in Dulbecco’s modified Eagle’s medium (Logan, UT) in the presence of 10% fetal bovine serum. MC3T3-E1 cells were maintained in α-minimum essential medium with 10% fetal bovine serum. Stable C2C12 cell lines were generated by transfection with the indicated expression vectors, followed by selection using G418-containing selection medium.

**Antibodies**—Anti-Myc (9E10) and anti-hemagglutinin (HA) (HA11.3) antibodies were purchased from Covance (Princeton, NJ). Horseradish peroxidase (HRP)-conjugated anti-FLAG (M2) (anti-FLAG-HRP) and anti-FLAG (M2) antibody-conjugated beads were from Sigma. Anti-cyclin D1, D2, D3, B, and A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-β-actin antibody was from Abcam (Cambridge, MA), and HRP-conjugated anti-mouse and anti-rabbit secondary antibodies were purchased from Pierce.

**Reagents**—For chromatin immunoprecipitation assays, single-stranded DNA and protein G-agarose beads were purchased from Sigma-Aldrich and Upstate Biotechnology, respectively. For the nonradioactive electrophoretic mobility shift assay (EMSA), we purchased a terminal deoxynucleotidyltransferase labeling kit and nonradioactive EMSA detection kit from Pierce. Terminal deoxynucleotidyltransferase labeling and EMSA were performed according to the manufacturer’s instructions.

**Plasmid Construction**—All full-length cDNAs were generated by PCR and subcloned into pcDNA3, for FLAG (M2) epitope fusion proteins, or pcDNA3.1, for HA or His\(_6\) epitope fusion proteins. All of the fusion proteins were generated as N-terminal epitope fusion proteins. Because there is only a single amino acid difference between mouse Msx2 and human Msx2 (there is a single amino acid deletion in the N terminus of mouse Msx2 as compared with human), we used mouse Msx2 for these experiments. However, we have used human MSX2 amino acid numbering to indicate the Msx2 mutation sites (the corresponding site of the P148H mutation of human MSX2 in mouse Msx2 would be P147H; to avoid confusion, we have maintained the human numbering system for the mouse cDNA). The cDNAs for Msx2, Praja1, and ubiquitin (Ub) from C2C12 cell were ligated into the BamHI and XhoI sites of pcDNA3 or pcDNA3.1 (Invitrogen) to generate pFLAG-Msx2-WT, pHA-Msx2-WT, pHis\(_6\)-Msx2-WT, pHA-Praja1, and pFLAG-Ub. To construct the expression plasmid for green fluorescent protein-conjugated Msx2 (pEGFP-Msx2), a BamHI/XhoI fragment of pFLAG-Msx2 was subcloned into the BglII and XhoI sites of pEGFP-C1 (Clontech). For the construction of bacterial expression vectors, BamHI and XhoI fragments of Msx2 and Praja1 were subcloned into the BamHI and SalI sites of pGEX6P-1 (Amersham Biosciences) and pET28a (Novagen), respectively, to generate pGEX-Praja1 and pET-Msx2, respectively. The mutant proteins Msx2 (P148H) and Msx2 (R172H) were generated by site-directed mutagenesis using pFLAG-Msx2-WT as the template and then subcloned into pET 28a and pcDNA3.1, respectively.

**In Situ Hybridization**—Probe preparation (Dlx5 and Msx2), tissue preparation, and in situ hybridization procedures were as described previously (28). Calvariae of ICR mice (embryonic days 12.5 and 15.5) were prepared as described previously (29). The sections were stained with hematoxylin and eosin to assess the developing calvaria. The 800-bp rat Dlx5 fragment in pcR2 (Invitrogen) was digested with BamHI and XbaI. The 850-bp murine Msx2 fragment in pSP72 (Invitrogen) was digested with HindIII and BglII. In all three cases, antisense and sense riboprobes were produced by T7 and SP6 RNA polymerases, respectively.

**Immunoblot and Immunoprecipitation**—The cell lysates were prepared from transfected cells using HEPES lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.25% sodium deoxycholic acid, 10% glycerol) supplemented with a protease inhibitor mixture (Roche Applied Science) and phosphatase inhibitors (Sigma-Aldrich). For immunoblot analysis, 25 μg of whole cell lysate were separated by 12% SDS-PAGE, and then the proteins were transferred electrophoretically onto a nitrocellulose membrane. Immunoreactive proteins were detected using a chemiluminescent detection kit. For immunoprecipitation analysis, 500 μg of precleared cell lysate were incubated with anti-FLAG (M2) antibody-conjugated beads or anti-HA antibodies and then incubated at 4 °C for 3 h with constant rotation. The beads were washed extensively five times, resuspended in Laemmli sample buffer, and then boiled at 100 °C for 3 min. Bound proteins were resolved by 12% SDS-
PAGE and analyzed by immunoblot using anti-FLAG-HRP and anti-HA antibodies.

**In Vivo Ubiquitylation Assay**—The cells were incubated in the presence of 10 μg/ml MG132. For the analysis of FLAG-Msx2-WT, endogenous Ub was detected using an anti-Ub antibody. For the detection of recombinant Ub, the cells were co-transfected with pFLAG-Ub and an expression vector for the indicated HA- or His-tagged target protein. In vivo ubiquitylation assays were carried out as previously described (30). Briefly, 1 × 10⁶ cells were lysed in 100 μl of TBS buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 2% SDS), boiled for 5 min, and briefly sonicated. The lysates were diluted with 900 μl of TXN dilution buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1% Triton X-100) and then subjected to centrifugation at 14,000 rpm at 4 °C for 45 min. Cleared lysate (1 mg) was subjected to immunoprecipitation with the indicated antibodies or an in vitro association (pull-down) assay using Ni²⁺-agarose in the presence of 10 mM imidazole. Enriched Ub-conjugated proteins were resolved by 7% SDS-PAGE and analyzed by immunoblot using anti-Ub or anti-FLAG-HRP antibodies.

**In Vitro Ubiquitylation Assay**—An in vitro ubiquitylation kit was purchased from BIOMOL, and the assay was carried out according to the manufacturer’s instructions. Full-length recombinant Mxs2 and Praja1 were generated using an in vitro transcription/translation kit (Promega) as follows. Transcription/translation reactions containing purified RNase-free pET28-Mxs2WT, pET28-Mxs2 (P148H), and pGEX/Praja1 were allowed to proceed at 37 °C for 30 min. Equal amounts of reaction mixture containing His-Mxs2-WT, His-Mxs2 (P148H), or glutathione S-transferase-Praja1 were then incubated with recombinant E1, E2, and biotinylated Ub in the presence and absence of the indicated reaction components. Ubiquitylated proteins were separated by SDS-PAGE and analyzed by immunoblot using anti-biotin antibody.

**Chromatin Immunoprecipitation**—The chromatin immunoprecipitation (ChIP) assay was performed as described previously (13). The cells were seeded in 100-mm dishes at a density of 1 × 10⁷ cells/dish and then transfected with expression plasmids for FLAG-Mxs2-WT and FLAG-Mxs2 (P148H). Twenty-four hours after transfection, the cells were treated with a protein synthesis inhibitor, cyclohexamide (CHX), in the presence or absence of the proteasome inhibitor MG132 for the indicated periods of time. For ChIP analysis, the following PCR primers were designed to amplify nucleotides −715 and −431 (relative to the mRNA cap site at +1) of the mouse Runx2 distal promoter: forward, 5′-AACACAGGGAAGCAGCCACC; and reverse, 5′-CCACACTCTCTGTTAGGTAAAGC.

**Electrophoretic Mobility Shift Assay**—EMSAs were performed using a nonradioactive EMSA kit and terminal deoxynucleotidyltransferase labeling kit from Pierce using the terminal deoxynucleotidyltransferase labeling kit. SaOS-2 cells were transiently transfected with expression vectors for FLAG-Mxs2-WT and FLAG-Mxs2 (P148H), and nuclear extracts were prepared 24 h post-transfection. The nuclear extracts were incubated with labeled double-stranded oligonucleotide in the absence or presence of a 200-fold molar excess of unlabeled competitor DNA for 20 min at room temperature. For the supershift assay, the nuclear extracts were preincubated with anti-FLAG antibodies (Sigma) for 20 min at room temperature before incubation with the labeled probe. Protein-DNA complexes were separated at 4 °C by 5% SDS-PAGE in the presence of 0.5× Tris-borate-EDTA and then analyzed by immunoblot assay. DNA-protein complexes are visualized by the detection of biotin-labeled DNA using anti-biotin antibody.

**Luciferase Reporter Activity Assay**—The cells were plated in 96-well plates at a density of 1 × 10⁴ cells/well. After overnight culture, the cells were transfected with expression vectors by electroporation using Microporator (Digital Bio Technology). Each transfection assay was performed with 0.2 μg/well of the Dlx5 and Mxs2 expression vectors or pcDNA3 and 0.2 μg of the ALP-luciferase reporter vector, ALP-553 (~553 to +81 bp), as previously described (14). After 24 h, the cells were harvested, and luciferase activity was measured using the BrightGlo luciferase assay kit (Promega, Madison, WI). Luminescence was recorded on a GloMax®-Multi Detection System (Promega).

**Cell Proliferation and Differentiation Assays**—Proliferation assays were carried out using a cell counting kit (CCK-8, Dojindo, Japan), according to the manufacturer’s instructions. The cells were seeded at a density of 1 × 10⁵ cells/well in a 96-well plate and allowed to incubate for 24 h. When the cells reached exponential growth phase, 10 μl of CCK-8 reagent was added to each well, and the cells were allowed to incubate for 90 min. The absorbance at 450 nm of each well was measured using a plate reader. C2C12 cells were transfected with pFLAG-Mxs2-WT or pFLAG-Mxs2 (P148H), and total cellular RNA was prepared using a total RNA isolation kit (Roche Applied Science), according to the manufacturer’s instructions. Reverse transcription was performed using a Superscript RT kit (Invitrogen), and PCR was carried out using the following primers for the amplification of FLAG and Mxs2 sequences: forward, 5′-ACTACAAGGACAGCATGACACAGAAGGATGAACTCCAG-3′ and reverse, 5′-CTCCTGTATCCAGTGCCTCG-3′. The levels of Mxs2 mRNA were normalized to those of glyceraldehyde-3-phosphate dehydrogenase, and relative mRNA expression levels are presented.

**RESULTS**

*Mxs2, but Not Mxs2 (P148H), Stimulates Cell Proliferation*—Previous studies have shown that there are multiple proliferation-related defects in cells of mesenchymal origin in Mxs2 knock-out (Mxs2−/−) mice (8). Transgenic mice that overexpress Mxs2 exhibit craniosynostosis because of the rapid proliferation of mesenchymal cells in osteogenic fronts and the subsequent rapid expansion of tissue. Using in situ hybridization, we found that Mxs2 is expressed in the cranial tissue of embryonic day 12.5 (E12.5) and E15.5 mouse embryos (Fig. 1A). Mxs2 mRNA expression was strong in the suture mesenchyme, where mesenchymal cells are actively proliferating to maintain suture patency. In coronal sections of E12.5 mouse heads, the expression patterns of Mxs2 and Dlx5 did not overlap. Mxs2 was expressed in the mesoderm of the primordial calvarium (arrowheads), whereas Dlx5 was expressed in the mesodermal area.
tissue of the mandible (mn), parts of the brain (b), and the mineralizing regions of the calvarium (arrows) (Fig. 1A). At E15.5, the stage at which skull mineralization occurs, Dlx5 mRNA was specifically expressed in the mineralized calvarium (arrows) and maxilla (mx), whereas Msx2 was detected only in the suture mesenchyme (arrowheads) (Fig. 1A). Thus, Msx2 expression was localized predominantly in the suture mesenchyme, and there was no overlap between Dlx5 and Msx2 expression. The observation that Dlx5 was expressed in mineralizing tissue, whereas Msx2 was expressed in as-yet unmineralized, proliferating primordial bone tissue implicates these genes in an antagonistic functional relationship that results in mutually exclusive expression patterns.

To determine the functional role of Msx2 in cellular proliferation, we examined the proliferation rate of C2C12 cells that were stably transfected with expression plasmids for Msx2-WT, Msx2 (P148H), antisense Msx2 (AS-Msx2), or Runx2. The rate of proliferation in Msx2-WT-transfected C2C12 cells was 1.8-fold higher than mock transfected cells. The expression of antisense Msx2 resulted in a lower rate of cell proliferation than Msx2-WT, and Runx2 stimulated cell proliferation, although not as strongly as Msx2-WT. The expression of Msx2 (P148H) failed to stimulate cell proliferation. These results indicated that Msx2-WT stimulates cell proliferation and that Msx2 (P148H) is functionally defective in this process.

To characterize the cell proliferation defect of Msx2 (P148H), we examined the expression of cyclin proteins in cells that overexpressed WT or mutant Msx2. Previously, it was reported that a cyclin D1/D2/D3 knock-out mouse has severe cell proliferation defects (31). The expression of FLAG-Msx2-WT increased the level of cyclin D family proteins in C2C12 cells, whereas FLAG-Msx2 (P148H) did not. Specifically, cyclin D1 and D2 were significantly increased by the expression of Msx2-WT or Msx2 (P148H) cell transformants. The levels of β-actin were also analyzed as a control for protein loading. D, the expression of endogenous Msx2 mRNA during cell cycle progression. The cells were synchronized by the removal of serum for 48 h, and cell cycle progression was triggered by the addition of serum. The cells were harvested at the indicated time points after serum stimulation, and mRNA was extracted. Flow cytometric analysis of cell cycle progression was determined using propidium iodide staining. The data were obtained for two independent experiments, with a minimum of 10,000 events recorded in each experiment. The mRNA levels were determined by reverse transcription-PCR.
of Msx2 gene expression in G1/S correlated with increased cyclin levels and the stimulation of cell cycle progression (Fig. 1D). These results suggested that the function of Msx2 is tightly linked to cell proliferation and that premature suture closure in Boston-type craniosynostosis may be due to a proliferation defect in Msx2 (P148H) mutation. This observation is particularly relevant, given that Msx2 is not only highly expressed in the suture mesenchyme in the early stages of craniofacial development but also strongly stimulates the proliferation pools of osteogenic front found in Msx2-transgenic mice.

Analysis of cells that overexpressed either EGFP-Msx2-WT or EGFP-Msx2 (P148H) by fluorescence microscopy revealed that there were marked differences in the signal intensities of the two proteins, which was consistent with the immunoblot results (Fig. 2F). Thus, the constitutively low level of expression of Msx2 (P148H) did not appear to be an experimental artifact but rather a true molecular phenotype of the mutant protein.

**Figure 2.** The expression of mutant Msx2 (P148H) protein is constitutively lower than Msx2-WT. A, C2C12 cells were transfected with expression vectors for FLAG- or Xpress-Msx2-WT or -Msx2 (P148H). The expression of WT and mutant proteins was analyzed by immunoblot 24 h after transfection using anti-FLAG or anti-Xpress antibodies. B, nucleotide sequence of the mutation site in Msx2-WT and Msx2 (P148H). C, reverse transcription-PCR analysis of the mRNA levels of overexpressed Msx2-WT and Msx2 (P148H). Forward and reverse primers were designed to amplify FLAG and Msx2 coding sequences. 26R and 28R indicate PCR cycle numbers. cDNA/RNase, control reaction using cDNA that was synthesized after RNase treatment; RNA, control reaction using RNA without reverse transcription. D, immunoblot analysis of Msx2-WT and Msx2 (P148H) in stable C2C12 transfectants. E, immunoblot analysis of Msx2 proteins in rat osteosarcoma ROS17/2.8 cells, osteoblast-like MC3T3-E1 cells, HEK293 cells, the human neural stem cell line NSC-F5, and SaOS-2 cells. F, the expression of EGFP-Msx2-WT and EGFP-Msx2 (P148H) in C2C12 cells was evaluated by fluorescence microscopy. The images were captured with an exposure time of 400 and 2800 ms, respectively.

Mutant Msx2 (P148H) Is Ubiquitinated at a Higher Level than Wild Type Msx2—To examine the half-life of mutant and wild type Msx2 proteins, we performed a pulse-chase experi-
MSX2-P148H Mutation Leads to Enhanced Degradation

A.

Chase (h)

|        | 0  | 0.5 | 1   | 2   | 4   | 8   |
|--------|----|-----|-----|-----|-----|-----|
| Mxs2-WT|    |     |     |     |     |     |
| Mxs2 (P148H)| |     |     |     |     |     |

B.

|        | WT | (P148H) |
|--------|----|---------|
| FLAG (WCL) |      |         |
| MG132 | +  |         |

IP: FLAG
IB: FLAG
β-actin (WCL)

C.

M.W. (kDa)

|        | 250 | 200 | 150 | 100 | 75  | 50  |
|--------|-----|-----|-----|-----|-----|-----|
| WT     |     |     |     |     |     |     |
| P148H  |     |     |     |     |     |     |

(Ub)n~
FLAG-Mxs2

IP: FLAG
IB: Ubiquitin

WCL

FLAG
β-actin

|        | 250 | 200 | 150 | 100 | 75  | 50  |
|--------|-----|-----|-----|-----|-----|-----|
| WT     |     |     |     |     |     |     |
| P148H  |     |     |     |     |     |     |

(Ub)n~
6xHIS-Mxs2

NTA purification
IB: FLAG

WCL

HIS
β-actin

NTA, nitrilotriacetic acid.

FIGURE 3. Mxs2 (P148H) is more susceptible to ubiquitin-dependent degradation in vivo that the WT protein. A, C2C12 cells were transfected with expression vectors for Mxs2-WT or Mxs2 (P148H). Twenty-four hours after transfection, the cells were metabolically labeled with [35S]methionine/[35S]cysteine for 2 h and then harvested after the indicated periods of time. Equal amounts of cell lysate were subjected to immunoprecipitation (IP) using anti-FLAG antibodies, and immune complexes were analyzed by SDS-PAGE, followed by autoradiography and densitometry. B, C2C12 cells were transfected with expression vectors for FLAG-Mxs2-WT or FLAG-Mxs2 (P148H), and total cell lysates were prepared 24 h after transfection. The lysates were subjected to immunoprecipitation using anti-FLAG antibody-conjugated beads, and immune complexes were analyzed by immunoblotting using HRP-conjugated anti-FLAG antibody. As controls, the level of Mxs2 in whole cell lysates (WCL) was determined using anti-FLAG antibodies, and the levels of β-actin were used to confirm equal protein loading. C, cells were transfected and harvested as described for B and then subjected to immunoprecipitation using anti-FLAG antibody. Immune complexes were analyzed by immunoblotting using anti-Ub antibody (top panel). C2C12 cells were co-transfected with expression vectors for FLAG-Ub and either His6-Mxs2-WT or His6-Mxs2 (P148H) for 24 h. The histidine fusion proteins were purified by Ni²⁺ column chromatography, and ubiquitin conjugation was assessed by immunoblot using an anti-FLAG antibody (lower panels).

Prajal Is the E3 Ligase That Is Responsible for Mxs2 Ubiquitination—E1, E2, and E3 are the major enzymes in ubiquitin-mediated protein degradation. E1 and E2 enzymes are involved in the degradation of most proteins, whereas E3 ligases are specific for each protein that is targeted for proteasomal degradation. Previously, it was reported that Prajal, a RING finger E3 ligase, is the E3 ligase for Mxs2 (30). The over-expression of Prajal in C2C12 cells strongly down-regulated mutant Mxs2 (P148H) and Mxs2-WT (Fig. 4A, upper panels) and was nearly undetectable at 8 h. Instead, Mxs2-WT was clearly detectable up to 8 h after metabolic labeling (Fig. 3A, lower panel). Densitometric analysis indicated that the half-life of Mxs2-WT is nearly four times longer than that of the mutant protein. These results suggested that the P148H mutation of Mxs2 results in an increased susceptibility of the protein to degradation.

Protein turnover in cells is regulated primarily by ubiquitination and proteasomal degradation. Intracellular ubiquitination is required for normal cellular homeostasis and is also involved in abnormal states of pathogenesis. To determine whether the decreased half-life of Mxs2 (P148H) was regulated by ubiquitination, we transfected C2C12 cells with expression vector for FLAG-Mxs2-WT or FLAG-Mxs2 (P148H) and then treated the cells with MG132, a potent inhibitor of the proteasome. MG132 treatment increased the protein levels of both Mxs2-WT and mutant Mxs2 (P148H) (Fig. 3B), but the effect of MG132 on Mxs2 (P148H) was more dramatic than its effect on Mxs2-WT. When we examined protein ubiquitination levels, we found that although both mutant and wild type proteins were ubiquitinated and underwent proteasomal degradation, the amount of ubiquitinated Mxs2 (P148H) was much higher than Mxs2-WT (Fig. 3C). These results were in good agreement with the results of the pulse-chase experiment (Fig. 1D). We also determined that Mxs2 (P148H) is more highly ubiquitinated than Mxs2-WT (Fig. 3D). These results indicated that the P148H mutation of Mxs2 renders the protein more susceptible to ubiquitin-dependent degradation.

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ment using [35S]Met/[35S]Cys metabolic labeling to analyze the de novo synthesis of exogenously expressed proteins. The half-life of mutant Mxs2 (P148H) was considerably shorter than Mxs2-WT. The level of FLAG-Mxs2 (P148H) was markedly decreased 4 h after metabolic labeling (Fig. 3A, lower panel) and was nearly undetectable at 8 h. In contrast, Mxs2-WT was clearly detectable up to 8 h after metabolic labeling (Fig. 3A, upper panels). Densitometric analysis indicated that the half-life of Mxs2-WT is nearly four times longer than that of the mutant protein. These results suggested that the P148H mutation of Mxs2 results in an increased susceptibility of the protein to degradation.

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ligases that are regulated by the bone morphogenetic protein/transforming growth factor-β signaling pathway. As shown in Fig. 4B, the expression of Praja1, but not Smurfl or Smurfl2, enhanced the degradation of both WT and mutant Msx2 proteins in a dose-dependent manner (Fig. 4C). Mutant Msx2 (P148H) appeared to be more susceptible to degradation than Msx2-WT, which indicated that the mutant protein is highly sensitive to degradation through E3 ligase targeting. These results implicated Praja1 as a candidate E3 ligase for the degradation of Msx2 and mutant Msx2 (P148H), because the level of degradation of both proteins appeared to be similar.

To examine Praja1-dependent ubiquitylation of Msx2 in more detail, we performed an in vitro ubiquitylation assay. His-tagged recombinant Msx2-WT and Msx2 (P148H) and glutathione S-transferase-tagged Praja1 were synthesized in vitro. In the absence of Mg2+ and ATP, or Praja1, there was no ubiquitylation of Msx2 proteins. In the presence of equal amounts of reaction components, the level of ubiquitin-conjugated Msx2 (P148H) was higher than Msx2-WT (Fig. 4D). The high molecular weight, ubiquitylated form of Msx2 appears as a diffuse band on an immunoblot. The band corresponding to ubiquitylated Msx2 (P148H) was more intense than that of Msx2-WT, which indicated that Msx2 (P148H) is more susceptible to Praja1-dependent ubiquitylation than Msx2-WT.

FIGURE 4. Praja1 is a candidate E3 ligase for Msx2 ubiquitylation. A–C, 293T cells were transiently cotransfected with expression vectors for FLAG-Msx2-WT or FLAG-Msx2 (P148H), and HA-Praja1, with or without MG132 treatment. The cells were lysed 24 h after transfection and analyzed by immunoblot using anti-FLAG and anti-HA antibodies. The membrane was rebotted with anti-β-actin antibodies to confirm equal protein loading. E, empty vector; P, Praja1; S1, Smurfl; S2, Smurfl2. D, in vitro ubiquitin conjugation using recombinant Msx2-WT and Msx2 (P148H), recombinant E1, E2, and E3 enzymes, biotin-labeled ubiquitin, and ATP in Mg2+-containing buffer, as indicated. Conjugation reactions were carried out at 37 °C for 30 min in a test tube, and then proteins were separated by SDS-PAGE and analyzed by immunoblot using anti-biotin antibody. GST, glutathione S-transferase.

Mss2 (P148H) Has Decreased Chromosome Binding Activity—Previously, it was reported that Msx2 specifically binds to an Msx2 response element in the Runx2-P1 promoter (13). We carried out an EMSA to examine the ability of wild type and mutant Msx2 to bind DNA. Msx2-WT was able to form complexes with synthetic oligonucleotides that contained the Msx2 response element of the Runx2-P1 promoter (Fig. 5A). Binding was inhibited by a 50-fold molar excess of unlabeled wild type oligonucleotide but not a mutant oligonucleotide (Fig. 5A). We also observed a shift in WT protein-DNA complexes when we preincubated the nuclear extracts with anti-FLAG antibodies (Fig. 5A). The level of WT and mutant protein-DNA complexes gradually increased with increasing amounts of biotin-labeled probe; however, the amount of mutant protein-DNA complexes was greater than WT protein-DNA complexes (Fig. 5B). In these experiments, we used nuclear extracts from SaOS-2 cells that were transiently transfected with expression plasmids for Msx2-WT and Msx2 (P148H) to ensure that comparable amounts of protein were present in each reaction (Fig. 5A, lower panel). Thus, these results indicated that the DNA binding affinity of Msx2 (P148H) is higher than that of Msx2-WT. Given that the ubiquitin-mediated degradation of mutant Msx2 is also higher than that of WT, we also examined the in vivo occupancy of the Msx2 cognate response element by either WT or mutant Msx2 using a ChIP assay. C2C12 cells were transfected with expression vectors for FLAG-Msx2-WT or FLAG-Msx2 (P148H) and then treated with CHX, a protein synthesis inhibitor, for various periods of time. Chromatin from the transfected cells was subjected to immunoprecipitation using an anti-FLAG antibody and then analyzed by PCR using primers that were specific for the Runx2 promoter region. The level of Runx2 distal promoter-bound Msx2 (P148H) decreased rapidly over time after CHX treatment. This rapid decrease in promoter occupancy was blocked by treatment with the proteasome inhibitor MG132 (Fig. 5C, upper panels). The level of promoter-bound Msx2-WT decreased less rapidly following CHX treatment and was not significantly affected by treatment with MG132 (Fig. 5C, lower panels). Fig. 5D shows that mutant Msx2 (P148H) is not able to suppress Dlx5-dependent transcriptional activity upon Alp promoter. It clearly demonstrates that the destabilization of Msx2 protein by P148H mutation brings about defective antagonizing activity of Msx2 to Dlx5. We also examined the local-
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A.

B.

C.

D.

E.

FIGURE 5. The in vitro DNA binding activity of Msx2 (P148H) is higher than WT protein, whereas in vivo binding activity is impaired. A and B, EMSA using FLAG-Msx2-WT or FLAG-Msx2 (P148H) and synthetic oligonucleotides that contained the homeodomain-specific response element of the murine Runx2 P1 promoter. Nuclear extracts (10 μg) from SaOS-2 cells that were transiently transfected with expression vectors for Msx2-WT (w) and Msx2 (P148H) (m) were incubated with biotinylated double-stranded oligonucleotides. The arrows indicate Msx2-DNA complex. C, ChIP analysis of the in vivo occupancy of the Runx2 P1 promoter. C2C12 cells were transfected with the indicated expression vectors, and 24 h after transfection, the cells were treated with 10 μg/ml CHX in the presence or absence of MG132. After the indicated periods of incubation, the cells were harvested and subjected to ChIP. D, transcriptional repression activity of wild type Msx2 and mutant Msx2 (P148H) against the activity of Dlx5 upon murine alkaline phosphatase promoter, respectively. C2C12 cells were transfected with the Alp-553 (~553 to +81 bp) promoter reporter constructs along with the Dlx5 and/or Msx2 expression vector. After the 24 h of transfection, luciferase activities were analyzed. E, C2C12 cells were transfected with expression vectors for EGFP-Msx2-WT or EGFP-Msx2 (P148H). Twenty-four hours after transfection, the cells were fixed, stained with DAPI to visualize the DNA, mounted, and analyzed by confocal microscopy. The green dots represent EGFP-Msx2-WT, the blue dots represent DAPI staining, and the white dots represent the co-localization of Msx2 and DNA. The signal intensities of EGFP-Msx2-WT (red line) and DAPI (blue line) from representative images are shown in the line plots.

In contrast, EGFP signals were very weak in cells that expressed Msx2 (P148H), indicating that the expression of the mutant protein was very low in these cells, and EGFP was nearly completely dissociated from DAPI signals, which indicated a very low level of protein-DNA interactions (Fig. 5E, lower panel). These results suggested that the in vivo chromosomal binding of Msx2 and the subsequent regulation of gene expression is...
regulated primarily by protein levels, not the binding activity of Msx2.

**Mutant Msx2 (P148H) Induces the Degradation of Wild type Msx2**—Our data suggested that MSX2 (P148H) is a loss-of-function allele because of the premature ubiquitylation and degradation of the mutant protein. In Boston-type craniosynostosis, because the second MSX allele is intact (i.e. WT), the mutation would represent a haploinsufficiency. However, in a known model of MSX2 haploinsufficiency, MSX2 (R172H), there is parietal foramina but no craniosynostosis (32). Moreover, MSX2 (P148H) is inherited as an autosomal dominant mutation. To begin to address this apparent discrepancy, we co-transfected C2C12 cells with expression plasmids for FLAG-Msx2-WT and either WT or mutant HA-Msx2 and examined the levels of ectopically expressed proteins by immunoblot. The level of FLAG-Msx2-WT was maintained when it was co-expressed with HA-Msx2-WT but was dramatically decreased when co-expressed with HA-Msx2 (P148H) (Fig. 6A). When HA-Msx2-WT was co-expressed with FLAG-Msx2 (P148H), the level of HA-Msx2-WT was dramatically decreased in a dose-dependent manner (Fig. 6B). The ubiquitylation of HA-Msx2-WT was higher in presence of co-expressed Msx2 (P148H) than Msx2-WT (Fig. 6C). These results indicated that the mutant form of Msx2 exerts a dominant negative effect on the WT protein. When we compared the effect of two different mutants of Msx2 (P148H) and Msx2 (R172H) on the ubiquitylation of Msx2-WT, we found that Msx2 (R172H), which is a loss-of-function mutant, had no effect on the stability of FLAG-Msx2-WT. The enhanced degradation and ubiquitylation of FLAG-Msx2-WT occurred only in the presence of Msx2 (P148H) (Fig. 6, D and E).

**DISCUSSION**

Boston-type craniosynostosis is due to a single amino acid substitution, P148H, in MSX2 (26). It has been widely accepted that P148H is a gain-of-function mutation that is responsible for the congenital abnormalities associated with the disease. There are two main lines of evidence for this assumption. First, the disease is a single allele mutation and is inherited in an autosomal dominant manner (26). In the case of single allele mutations, the disease phenotype of a loss-of-function mutation is usually not transmitted in a dominant fashion, because the second, normal allele carries out the proper function of the gene. Diseases that are transmitted in a dominant manner are usually gain-of-function mutations. Second, mutant MSX2 (P148H) has a much stronger DNA binding activity than the WT protein. Because MSX2 is a transcription factor, DNA binding is an important aspect of its function. In the current study, we have demonstrated that MSX2 (P148H) is a loss-of-function mutant and is more susceptible to ubiquitin-mediated degradation than the WT protein. Moreover, mutant Msx2 strongly enhanced the degradation of Msx2-WT when the two proteins were co-expressed in cells. Thus, our data indicate that Msx2 (P148H) has a dominant-negative effect on Msx2 and provide an explanation for why a loss-of-function mutation exhibits a dominant pattern of inheritance. We also showed that Msx2 (P148H) has a higher DNA binding activity (27) than Msx2-WT (Fig. 5B); however, the increased DNA binding activity in vitro does not correlate with in vivo chromosomal occupancy. The lower in vivo chromosomal occupancy of Msx2 (P148H) is most likely due to its increased ubiquitin-dependent degradation (Fig. 5C).

Until recently, there have been few reports on the post-translational modification of Msx2. It has been shown that the adaptor molecule Dlxin-1 mediates the ubiquitylation of Msx2 (30). In the current study, we demonstrated that ubiquitylation is a critical post-translational modification of Msx2. We demonstrated that the ubiquitin-dependent degradation of Msx2 is crucial for Msx2 protein turnover and that Praja1 is the primary E3 ligase involved in this process. Importantly, we have shown that Msx2 (P148H) is much more susceptible to ubiquitin-dependent degradation than Msx2-WT and that the mutant protein enhances the ubiquitylation of the WT protein. Although the mechanism of enhanced ubiquitylation of Msx2-WT by
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Msx2 (P148H) is not clear, it is possible that protein-protein interactions between WT and mutant proteins results in the destabilization of Msx2-WT. In fact, we have shown that the WT protein is specifically degraded in the presence of the P148H mutant when the two proteins are co-expressed in cells. Of note, the co-expression of another Msx2 mutant, Msx2 (R172H), which exhibits reduced DNA binding activity and causes parietal foramina, did not affect the turnover of Msx2-WT. Thus, whereas both mutants appear to be loss-of-function mutants, the mechanism of impaired function appears to be quite different for the two proteins. This may also explain why the two mutations result in different phenotypes. Arginine 172 is located in helix 3 of the homeodomain in MSX2 and has been shown to be critical for the interaction of MSX2 with the phosphate of the DNA backbone (32). When Msx2 (P148H) was co-expressed with WT protein, both the mutant and WT proteins disappeared rapidly. In contrast, the turnover rate of Msx2 (R172H) was similar to Msx2-WT. These results indicate that although a sustained level of Msx2 (R172H) may be sufficient to counteract the binding of the Dlx5 to its cognate response element, it is likely able to counteract Runx2 or Dlx5 through protein-protein interactions.

What then is the function of MSX2 in cranial suture closure? Previous reports have indicated that Msx2 plays a critical role in the proliferation of osteoprogenitor cells in the osteogenic fronts of the suture area (22, 33). The cranial suture is a mass of soft tissue between two calvarial bones and is the growing front of the calvarial flat bones. Therefore, maintenance of the suture space by the continuous recruitment of new osteoprogenitor cells is crucial for the expansion of the calvarial bones as the brain and face grow. In the current report, we have demonstrated that the overexpression of Msx2 stimulates cell proliferation by ~80%, whereas Msx2 (P148H) does not. The stimulation of cell proliferation may be due in part to an increase in cyclin D levels in Msx2 overexpressing cells, because Msx2 (P148H) failed to induce cyclin D expression. The inability of Msx2 (P148H) to induce cyclin D expression may be due to the preferential and rapid breakdown of the mutant protein.

The current results showed that just a transient transfection of Msx2 dramatically increased cyclin D level as shown in Fig. 2C, suggesting its critical role as a positive regulator in the maintenance of cyclin D levels. Cyclin D is very well known key molecule for the cell cycle progression in normal and tumor cells (31). In this study we have tested Msx2 protein stability in several different cell lines (Fig. 2E). Msx2-WT protein showed consistently higher stability than Msx2 (P148H) in most of the normal mesenchymal cell lines. Very interestingly, different results came out from human osteosarcoma cell lines of different genetic background. SaOS-2 cells are deficient of p53 and Rb function (34). HOS cells are deficient of p53 by the mutation of both alleles but have an intact Rb gene (35). Meanwhile, U2OS cells have both intact Rb and p53 genes (36). The decreased stability of Msx2 (P148H) in U2OS cells is very similar to that determined in normal cells. In contrast, in SaOS-2 and HOS cells the protein stability of Msx2 (P148H) does not change significantly. These results suggest that Msx2 (P148H) stability change is closely related with p53 or its downstream gene function. However, Rb involvement probably is ruled out because Rb-proficient HOS cells also showed stable Msx2 (P148H). In addition, it is widely known that Rb activity is controlled by CDKs and cyclin D1 (37). In this paper we showed that cyclin D1 is again controlled by Msx2. Thus, Msx2 could be a more upstream regulator of Rb action.

The regions of expression of Msx2 and Dlx5 are mutually exclusive in the developing cranial suture (Fig. 1A), and it has been shown that these two homeodomain proteins suppress the expression of the other (9, 10, 14, 15). We have previously shown that the expression of alkaline phosphatase and Runx2 is down-regulated by Msx2 through the inhibition of Dlx5 function (13, 14), and others have reported that Msx2 can counteract the activity of osteogenic transcription factors through protein-protein interactions (9–11, 15). The results of the current study build on these earlier reports and suggest that the down-regulation of Msx2 through the dominant negative action of Msx2 (P148H) alleviates the suppression of Dlx5, resulting in a more rapid invasion of the suture space by mineralized areas and premature suture obliteration. The notion is further supported by the Alp promoter reporter assay, which showed that Msx2 (P148H) could not suppress Dlx5 stimulated Alp promoter activity, whereas wild type Msx2 did (Fig. 5D). In addition, the P148H mutant could influence on the destabilization of Msx2-WT protein but could not perform a dominant-negative action on the Dlx5 protein turnover. These results indicate that other Msx2 (P148H)-mediated protein degradation looks quite specific for the Msx2 protein. It has also been reported that bone thickness is generally decreased, and there are multiple hypoplastic areas in the endocranial side of the calvarium in Boston-type craniosynostosis patients (24) that may also reflect a deficiency in the recruitment of osteoprogenitor cells and subsequent premature mineralization caused by the premature degradation of MSX2.

In summary, premature suture closure in Boston-type craniosynostosis results from the enhanced ubiquitin-dependent degradation of MSX2, which functions in the maintenance of the suture space through the stimulation of osteoprogenitor cell proliferation and the inhibition of mineralization.

REFERENCES

1. Ryoo, H. M., Lee, M. H., and Kim, Y. J. (2006) Gene (Amst.) 366, 51–57
2. Liu, Y. H., Ma, L., Wu, L. Y., Luo, W., Kundu, R., Sangiorgi, F., Snead, M. L., and Maxson, R. (1994) Mech. Dev. 48, 187–197
3. Sumoy, L., Wang, C. K., Lichtler, A. C., Pierro, L. J., Kosher, R. A., and Upholt, W. B. (1995) Dev. Biol. 170, 230–242
4. Nishikawa, K., Nakamichi, T., Aoki, C., Hattori, T., Takashashi, K., and Taniguchi, S. (1994) Biochem. Mol. Biol. Int. 32, 763–771
5. Koshiba, K., Kuroiwa, A., Yamamoto, H., Tamura, K., and Ide, H. (1998)J. Exp. Zool. 282, 703–714
6. Ros, M. A., Lyons, G. E., Mackem, S., and Fallon, J. F. (1994)Dev. Biol. 166, 59–72
7. Wang, W. P., Widelitz, R. B., Ji, T. X., and Chuong, C. M. (1999)J. Investig. Dermatol. 4, 278–281
8. Sakotaka, I., Ma, L., Ohshima, H., Bei, M., Woo, I., Nishizawa, K., Maeda, T., Takano, Y., Uchiyama, M., Heaney, S., Peters, H., Tang, Z., Maxson, R., and Maas, R. (2000)Nat. Genet. 24, 391–395
9. Zhang, H., Hu, G., Wang, H., Sciavolino, P., Iler, N., Shen, M. M., and Abate-Shen, C. (1997) Mol. Cell. Biol. 17, 2920–2932
10. Newberry, E. P., Latifi, T., and Towler, D. A. (1998)Biochemistry (Mosc.) 37, 16360–16368
11. Newberry, E. P., Latifi, T., Batteia, J. T., and Towler, D. A. (1997)Biochem-
istry (Mosc.) 36, 10451–10462
12. Newberry, E. P., Boudreaux, J. M., and Towler, D. A. (1997) J. Biol. Chem. 272, 29607–29613
13. Lee, M. H., Kim, Y. J., Yoon, W. J., Kim, J. I., Kim, B. G., Hwang, Y. S., Wozney, J. M., Chi, X. Z., Bae, S. C., Choi, K. Y., Cho, J. Y., Choi, J. Y., and Ryoo, H. M. (2005) J. Biol. Chem. 280, 35579–35587
14. Kim, Y. J., Lee, M. H., Wozney, J. M., Cho, J. Y., and Ryoo, H. M. (2004) J. Biol. Chem. 279, 50773–50780
15. Hassan, M. Q., Javed, A., Morasso, M. I., Karlin, J., Montecino, M., van Wijnen, A. J., Stein, G. S., Stein, J. L., and Lian, J. B. (2004) Mol. Cell Biol. 24, 9248–9261
16. Yoshizawa, T., Takizawa, F., Iizawa, F., Ishibashi, O., Kawashima, H., Matsuda, A., Endo, N., and Kawashima, H. (2004) Mol. Cell Biol. 24, 3460–3472
17. Cheng, S. L., Shao, J. S., Charlton-Kachigian, N., Loewy, A. P., and Towler, D. A. (2003) J. Biol. Chem. 278, 45969–45977
18. Towler, D. A., Shao, J. S., Cheng, S. L., Pingsterhaus, J. M., and Loewy, A. P. (2006) Ann. N. Y. Acad. Sci. 1068, 327–333
19. Shao, J. S., Cheng, S. L., Pingsterhaus, J. M., Charlton-Kachigian, N., Loewy, A. P., and Towler, D. A. (2005) J. Clin. Investig. 115, 1210–1220
20. Wang, J. C., Steinraths, M., Dang, L., Lomax, B., Eydoux, P., Stockley, T., Yong, S. L., and Van Allen, M. I. (2007) Am. J. Med. Genet. A 143, 2931–2936
21. Shihara, T., Kato, M., Kimura, T., Hayasaka, K., Yamamori, S., and Ogata, T. (2004) Am. J. Med. Genet. A 128, 214–216
22. Liu, Y. H., Tang, Z., Kundu, R. K., Wu, L., Luo, W., Zhu, D., Sangiorgi, F., Sneed, M. L., and Maxson, R. E. (1999) Dev. Biol. 205, 260–274
23. Dodig, M., Tadic, T., Kronenberg, M. S., Dacic, S., Liu, Y. H., Maxson, R., Rowe, D. W., and Lichtler, A. C. (1999) Dev. Biol. 209, 298–307
24. Warman, M. L., Mulliken, J. B., Hayward, P. G., and Muller, U. (1993) Am. J. Med. Genet. 46, 444–449
25. Liu, Y. H., Kundu, R., Wu, L., Luo, W., Ignelzi, M. A., Jr., Sneed, M. L., and Maxson, R. E., Jr. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6137–6141
26. Jabs, E. W., Muller, U., Li, X., Ma, L., Luo, W., Haworth, I. S., Klisak, I., Sparkes, R., Warman, M. L., Mulliken, J. B., Sneed, M. L., and Maxson, R. (1993) Cell 75, 443–450
27. Ma, L., Golden, S., Wu, L., and Maxson, R. (1996) Hum. Mol. Genet. 5, 1915–1920
28. Park, M. H., Shin, H. I., Choi, J. Y., Nam, S. H., Kim, Y. J., Kim, J. H., and Ryoo, H. M. (2005) J. Bone Miner. Res. 16, 885–892
29. Kim, H. J., Rice, D. P., Kettunen, P. J., and Thesleff, I. (1998) Development 125, 1241–1251
30. Sasaki, A., Masuda, Y., Ikeda, K., and Watanabe, K. (2002) J. Biol. Chem. 277, 22541–22546
31. Kozar, K., Ciemerych, M. A., Rebel, V. I., Shigematsu, H., Zagozdzon, A., Sicinski, E., Geng, Y., Yu, Q., Bhattacharya, S., Bronson, R. T., Akashi, K., and Sicinski, P. (2004) Cell 118, 477–491
32. Wilkie, A. O., Tang, Z., Elanko, N., Walsh, S., Twigg, S. R., Hurst, I. A., Wall, S. A., Chrzansowska, K. H., and Maxson, R. E., Jr. (2000) Nat. Genet. 24, 387–390
33. Rice, R., Rice, D. P., Olsen, B. R., and Thesleff, I. (2003) Dev. Biol. 262, 75–87
34. Shew, J. Y., Lin, B. T., Chen, P. L., Tseng, B. Y., Yang-Feng, T. L., and Lee, W. H. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 6–10
35. Romano, J. W., Ehrhart, J. C., Duthu, A., Kim, C. M., Appella, E., and May, P. (1989) Oncogene 4, 1483–1488
36. Montanaro, L., Mazzini, G., Barbieri, S., Vici, M., Nardi-Pantoli, A., Govoni, M., Donati, G., Trere, D., and Derenzini, M. (2007) Cell Prolif. 40, 532–549
37. Burkhart, D. L., and Sage, J. (2008) Nat. Rev. Cancer 8, 671–682