pRB family proteins are required for H3K27 trimethylation and Polycomb repression complexes binding to and silencing p16\(^{INK4a}\) tumor suppressor gene

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Genetic studies have demonstrated that Bmi1 promotes cell proliferation and stem cell self-renewal with a correlating decrease of p16\(^{INK4a}\) expression. Here, we demonstrate that Polycomb genes EZH2 and BMI1 repress p16 expression in human and mouse primary cells, but not in cells deficient for pRB protein function. The p16 locus is H3K27-methylated and bound by BMI1, RING2, and SUZ12. Inactivation of pRB family proteins abolishes H3K27 methylation and disrupts BMI1, RING2, and SUZ12 binding to the p16 locus. These results suggest a model in which pRB proteins recruit PRC2 to trimethylate p16, priming the BMI1-containing PRC1L ubiquitin ligase complex to silence p16.

Received October 3, 2006, revised version accepted November 14, 2006.

The mammalian pRB family proteins, pRB, p107, and p130 (also known as pocket proteins), play a key role in controlling the G1-to-S transition of the cell cycle and maintaining differentiated cells in a reversible quiescent or permanent senescent arrest state (Weinberg 1995; Czabotar 2005). The pocket proteins are hypophosphorylated in cells exiting mitosis as well as in quiescent cells, where they bind to and negatively regulate the function of the E2F family transcription factors (Trimarchi and Lee 2002). In cells entering the cell cycle, extracellular mitogens first induce the expression of D-type cyclins, which bind to and activate CDK4 and CDK6, leading to the phosphorylation of pRB family proteins, causing functional inactivation by E2F dissociation, thereby promoting a G1-to-S transition. Inhibition of CDK4 and CDK6 by the INK4 family of CDK inhibitors (p16\(^{INK4a}\), p15\(^{INK4b}\), p18\(^{INK4c}\), and p19\(^{INK4d}\)) retains pRB family proteins in their hypophosphorylated, growth-suppressive states and prevents G1-to-S progression. Disruption of the INK4–RB pathway, consisting of INK4–cyclinDs–CDK4/6–RB–E2Fs, deregulates G1-to-S control and represents a common event in the development of most, if not all, types of cancer (Sherr 1996).

Among the major challenges toward a better understanding of G1 control by the INK4–pRB pathway is how different INK4 genes are regulated, thereby linking G1 control to different cellular pathways. INK4 proteins are relatively stable, and the primary regulation of INK4 is through transcriptional control. The expression of each of the INK4 genes is distinctly different during development, in different adult tissues, and in response to different cellular conditions (Roussel 1999). There have been only a few reports wherein a transcriptional regulator has been demonstrated to bind to an INK4 promoter by either gel shift or chromatin immunoprecipitation (ChIP) assay. Identification of factors that directly bind to INK4 promoters holds the key to linking different cellular pathways to G1 control by the INK4–pRB pathway, but these links remain disproportionately poorly understood in comparison with our knowledge of the function of the INK4–pRB pathway (Pei and Xiong 2005).

To elucidate the molecular mechanisms regulating p16 expression, we tested whether p16 gene expression is directly regulated by BMI1, an oncogene that encodes a transcriptional repressor of the Polycomb group (PCG) of proteins (van Lohuizen et al. 1991; Lund and van Lohuizen 2004). Deletion of Bmi1 retards cell proliferation, causes premature senescence in mouse embryonic fibroblasts (MEFs), and reduces the number of hematopoietic stem cells, with an associated up-regulation of p16 (and to a lesser extent of p19\(^{Arf}\)). Codeletion of p16 (or p16\(^{Arf}\)) partially rescued the proliferative defects of Bmi1-null cells (Jacobs et al. 1999a; Brugemann et al. 2005; Molosky et al. 2005), providing genetic evidence supporting a functional interaction between the Bmi1 and p16 genes. However, whether BMI1 directly binds to and regulates the transcription of the p16 gene has not been demonstrated. A notable feature of p16 is its high level of expression in virally transformed cells (Xiong et al. 1993) and its inverse correlation with pRB function (Tam et al. 1994; Har et al. 1996), suggesting a negative regulation of p16 gene expression by pRB (Li et al. 1994). We therefore also examined whether pRB and BMI1 collaboratively repress p16 expression.

Results and Discussion

pRB family proteins negatively regulate p16 gene expression

The p16 protein is expressed at a high level in DNA tumor virus-transformed cells, including VA13 cells, that were transformed from normal human fibroblasts (WI38 cells) by SV40 (Fig. 1A; Xiong et al. 1993). The high level of p16 protein in VA13 cells was correlated with a high level of p16 mRNA (Fig. 1B), indicating transcriptional activation of the p16 gene. We established WI38 stable cell lines expressing either type 16 papilloma viral oncoprotein E6 (inactivating p53) or E7 (inactivating pRB family proteins). To avoid the possible accumulation of
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Figure 1. pRB family proteins negatively regulate p16 gene expression. The levels of p16, p18, and p21 proteins [A,C] and mRNA [B,D] were determined in WI38 and VA13 cells by direct immunoblotting or Q-RT–PCR. Data are expressed relative to the corresponding values for WI38 [B] or WI-38/Mock [D] cells, and mean values and standard deviations were calculated from triplicates of a representative experiment. [E] WI38 cells were infected with an empty lentivirus vector [Mock] or lentivirus vectors encoding shRNA silencing individually human RB, p107, or p130 genes. The efficiency of silencing and the effect of silencing on p16 expression were determined by direct immunoblotting.

Additional genetic changes that might occur during the extended life span of WI38 cells after E6 and/or E7 expression, we used WI38/E6 or WI38/E7 stable lines within 10 passages, well before they reach a senescent stage. Compared with parental WI38 cells, both p16 protein and mRNA were slightly increased in WI38/E6 cells and substantially increased in WI38/E7 cells [Fig. 1C,D], confirming the inverse correlation between p16 gene expression and pRB function. The pRB family includes three pocket proteins, pRB, p107, and p130, with pRB specifically binding to E2F1/2/3/4 and p107 and p130 to E2F4/5 (Trimarchi and Lees 2002). Previous studies have used either virally transformed cells in which all three RB family genes are functionally inactivated or tumor-derived cells that have accumulated additional mutations, making it unclear which pRB family protein(s) may be involved in repressing p16 expression. To determine this, we infected normal WI38 human primary fibroblasts with lentiviruses encoding short hairpin RNA (shRNA) molecules silencing each individual RB family gene and determined the consequences of this knockdown on the level of p16 expression. Silencing of each gene was verified by direct Western blot and resulted in an increase of p16 expression [Fig. 1E]. These data indicate that all three pRB family proteins are normally involved in repressing p16 expression.

The oncogene BMI1 represses p16 expression

To search for the mechanism underlying the repression of p16 by the pRB family proteins, we examined the role of BMI1 in p16 regulation. BMI1 ectopic expression extends the replicative life span of normal human fibroblasts and is associated with a decrease in p16 expression [Jacobs et al. 1999a; Itahana et al. 2003]. We first examined whether BMI1 also represses other INK4 genes. A WI38 stable cell line ectopically expressing BMI1 was established after retroviral transduction and was used to examine the expression of all four INK4 genes. Confirming the negative regulation of p16 by BMI1, quantitative RT–PCR (Q-RT–PCR) analysis showed that the level of p16 mRNA in WI38/BMI1 cells was substantially decreased, to <20% of that of cells infected with empty virus [WI38/Mock] [Fig. 2A]. Associated with p16 mRNA decrease, the steady-state level of p16 protein was also decreased substantially in WI38/BMI1 cells [Fig. 2B]. The level of p15INK4b mRNA decreased by ~50% in WI38/BMI1 cells, while p16INK4d and p14ARF expression was not significantly affected by ectopic BMI1 expression [Fig. 2A]. Unexpectedly, p18INK4c mRNA was increased in WI38 cells expressing BMI1. The increase of p18INK4c mRNA was associated with an increase in the steady-state level of p18INK4c protein [Fig. 2B]. The mechanism underlying the BMI1-mediated p18INK4c increase is not clear at present.

We next examined conversely how loss of BMI1 function affects INK4 gene expression. We designed a retroviral vector encoding a shRNA that specifically targets human BMI1. Infection of WI38 cells with BMI shRNA retroviruses reduced BMI1 level to an undetectably low level [Fig. 2C]. Associated with BMI1 decrease is a substantial increase of p16 protein [Fig. 2C]. Q-RT–PCR analysis showed that of the four INK4 and ARF genes, only p16 mRNA was significantly increased in WI38 cells when BMI1 was silenced [Fig. 2D]. The level of p18INK4c and p14ARF mRNA was actually decreased slightly by BMI1 silencing. Associated with p16 in-

Figure 2. The oncogene BMI1 represses p16 gene expression. [A,B] WI38 cells were infected with empty [Mock] or BMI1-expressing retroviruses and selected by puromycin treatment. The levels of individual mRNA [A] and protein [B] levels were determined by Q-RT–PCR and direct immunoblotting, respectively. Q-RT–PCR results are expressed relative to the corresponding values for WI38/Mock cells, and mean values and standard deviations were calculated from triplicates of a representative experiment. [C] WI38 cells were infected with a retrovirus vector encoding shRNA against either GFP or BMI1 and selected by puromycin treatment. The efficiency of BMI1 silencing and the effect of BMI1 silencing on p16 expression were determined by direct immunoblotting. [D] The effects of BMI1 silencing on the expression of the four INK4 and ARF genes were determined by Q-RT–PCR, and results are expressed relative to the corresponding values for WI38/GFP-i cells. The mean values and standard deviations were calculated from triplicates of a representative experiment. [E] The growth curves of WI38 cells infected with a retrovirus vector encoding shRNA against either GFP or BMI1. Viable cells were counted by Trypan Blue staining at indicated days after initial seeding of 2 × 10^5 cells.
crease, BMI1 knockdown resulted in slower cell growth (Fig. 2E). Together, these results demonstrate that of the four INK4 and ARF genes, p16 seems to be a specific target of BMI1 function in human primary cells. In Bmi1−/− MEFs, both p16 and Arf mRNA levels are increased [Jacobs et al. 1999a,b], suggesting that the regulation of the mouse p16–Arf locus by Bmi1 is different from that of the human locus.

**BMI1-mediated p16 repression requires the function of pRB proteins**

Both BMI1 and pRB family proteins repress p16 expression, which led us to determine whether they may functionally cooperate. We first infected WI38 primary fibroblasts, VA13, 293 cells that were transformed by adenovirus [inactivating pRB family proteins by E1A], and Saos-2 osteosarcoma cells that contain a mutation in the RB gene with retroviruses expressing BMI1. In contrast to p16 repression by BMI1 in WI38 cells, ectopic BMI1 expression had no effect on p16 expression in VA13, 293, and Saos-2 cells despite high levels of p16 expression in these cells [Fig. 3A]. Consistent with the notion that BMI1 promotes cell proliferation in part through the repression of p16, transduction by the BMI1 retroviruses increased the proliferation rate of WI38 cells but had no effect on the proliferation of VA13 and Saos-2 cells [Fig. 3B].

VA13, 293, and Saos-2 cells have sustained additional genetic changes or functional loss caused by viral transduction in addition to the inactivation of the RB gene or pRB family function. To confirm that the function of pRB family proteins is required for p16 repression by BMI1, we infected WI38/E7 cells with a retrovirus expressing BMI1 and compared the level of p16 with WI38 cells infected with control empty retrovirus. In spite of a high level of p16 in WI38/E7 cells, ectopic expression of BMI1 had no detectable effect on p16 protein level [Fig. 5C]. Confirming this result, Q-RT–PCR showed that ectopic BMI1 expression reduced p16 mRNA by ∼75% in WI38/Mock cells but had very little effect in WI38/E7 cells (<10% repression) [Fig. 3D]. SUZ12, BMI1, and RING1 bind to the HoxC13 locus and repress its expression in HeLa cells deficient for pRB protein function [Cao et al. 2005], and the expression of HoxD10, another target gene of Bmi1-deficient cells, remains unchanged after E7 expression, indicating that the link between pocket proteins is not broadly involved in the regulation of PcG target genes. Taken together, these data indicate that pRB family proteins are required for BMI1-mediated repression of p16 expression.

**BMI1 binds to the p16 genomic region in an RB-dependent manner**

Previous genetic studies in mice have linked the function of Bmi1 in controlling cell proliferation and stem cell renewal with repression of p16 [Jacobs et al. 1999a; Smith et al. 2003]. Whether Bmi1 binds directly to and regulates the transcription of the p16 gene has not been tested yet. To clarify this issue, we carried out ChIP assays to test whether Bmi1 directly binds to the p16 gene. Oligonucleotide primers were designed corresponding to a sequence within mouse p16 exon 1a and exon 2, where it was recently shown to be bound by the Phc2 protein [Isono et al. 2005], a member of the PRC1L [human Polycomb-repressive complex-1-like] E3 ubiquitin ligase complex, which also includes RING1, RING2, and BMI1 [H. Wang et al. 2004]. A ChIP assay demonstrated that Bmi1 binds directly to p16 exon 1a and exon 2 in early-passage young MEF cells [Fig. 4A, right panel, lane 3], providing the first direct evidence that Bmi1 regulates p16 expression by directly binding to regulatory sequences of the p16 gene. The p16 protein level increased during in vitro passage of MEFs, and contributes at least in part to cellular senescence. Concomitant with the increase in the p16 mRNA level, the Bmi1 protein level decreased in a passage-dependent manner [Fig. 4A, left panel]. Consistent with this observation, the binding of Bmi1 to the p16 gene also decreased in a passage-dependent manner [Fig. 4A, right panel, lanes 3, 6, 9]. We noted that the decrease of Bmi1 binding to p16 preceded the decrease of Bmi1 level [Fig. 4A, passage 5], suggesting that Bmi1 level may not be the rate-limiting factor in p16 repression and another factor(s) may be involved.

To determine how the function of pRB family proteins may affect the regulation of p16 by Bmi1, we carried out ChIP assays in parallel in both wild-type MEFs and MEFs triply deficient for all three pRB proteins (TKO). The expression of pRB, p107, and p130 is readily detected in young MEF cells [Fig. 4B, right panel, lane 3], providing the first direct evidence that Bmi1 regulates p16 expression by directly binding to regulatory sequences of the p16 gene. The p16 protein level increased during in vitro passage of MEFs, and contributes at least in part to cellular senescence. Concomitant with the increase in the p16 mRNA level, the Bmi1 protein level decreased in a passage-dependent manner [Fig. 4A, left panel]. Consistent with this observation, the binding of Bmi1 to the p16 gene also decreased in a passage-dependent manner [Fig. 4A, right panel, lanes 3, 6, 9]. We noted that the decrease of Bmi1 binding to p16 preceded the decrease of Bmi1 level [Fig. 4A, passage 5], suggesting that Bmi1 level may not be the rate-limiting factor in p16 repression and another factor(s) may be involved.

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Previous studies on the silencing of Hox genes by the PcG proteins suggest a hierarchical recruitment model.
wherein PRC2-mediated H3K27 methylation is required for recruitment of PRC1L, which causes H2A-K119 ubiquitination both in fly and human cells (L. Wang et al. 2004; Cao et al. 2005). Therefore, we examined whether EZH2, a catalytic component of PRC2, is also required to repress p16 expression. Western blotting and Q-RT–PCR analysis showed that both p16 protein and mRNA were significantly increased in WI38 cells when EZH2 was silenced (Fig. 4C), suggesting that PRC2 is also required for the p16 repression.

We next examined BMI1 association with the human p16 gene. For this purpose, we designed a panel of >30 pairs of primers covering a 8-kb region on both sides of human p16 exon 1α and searched for the BMI1-binding site (data not shown). Positive binding of BMI1 was detected in a sequence within the human promoter close to the 5′ end of exon 1α and the intron between exon 1α and exon 2 in WI38 cells [Fig. 4D, lanes 2,3]. We also demonstrated that RING2, a component of the hPRC1L complex, and SUZ12, a component of PRC2, also bind to the p16 gene [Fig. 4D, lanes 2,3], further supporting the notion that PRC2 and hPRC1L directly regulate p16.

Given that the RB genes are required for BMI1 recruitment (Fig. 4B), we investigated whether they are also required for PRC2 binding to and H3K27 methylation of the p16 locus. As determined by ChIP assay, the p16 locus is bound by SUZ12 and is methylated at Lys27 of H3 [Fig. 4D, lanes 1–3]. Importantly, inactivation of the pRB family proteins by the expression of E7 completely abolished the SUZ12 binding to and H3K27 methylation of the human p16 gene and disrupted the binding of BMI1 and RING2 to the p16 locus [Fig. 4D, lanes 6,7]. H3K4 methylation, on the other hand, is slightly increased in WI38/E7 cells (data not shown), indicating that not all histone methylation marks on the p16 locus are affected the same way by the inactivation of pocket proteins. These results suggest that pRB proteins function upstream of both the PRC2 and PRC1L complexes.

The results presented here provide the first biochemical evidence supporting a direct regulation of p16 transcription by the PRC2 histone methyltransferase complex and the BMI1–RING2-containing PRC1 histone ubiquitin ligase complex. We further demonstrated that both H3K27 methylation at and BMI1/RING2 binding to the p16 locus require the function of the pRB family proteins, linking for the first time H3K27 methylation and the function of BMI1 with the pRB proteins. The detailed biochemical mechanism by which pRB family proteins collaborate with BMI1 to repress p16 transcription is yet to be determined. In repeated attempts, we have not been able to detect binding of pRB to the p16 locus. The simplest model suggested by our results is that the pRB family proteins are either involved in regulating the enzymatic activity or the recruitment of PRC2 to the p16 locus. H3K27 methylation by PRC2 would then facilitate recruitment of the BMI1-containing PRC1L complex to ubiquitinate H2A, leading to p16 silencing.

Our results also suggest a regulatory loop between p16 and the pocket proteins, with p16 acting as an upstream activator of the pocket proteins and the pocket proteins repressing p16 transcription as negative feedback. INK4 proteins are intrinsically stable and, once synthesized,
stably bind to and inhibit the activity of CDK4/6 by both interfering with ATP binding and by reducing the cyclin–CDK4/6 surface [Jeffrey et al. 2000]. Without a mechanism for repressing INK4 expression, mitogen-induced cyclin D synthesis would not be able to offset INK4 from CDK4/6, and, displaced, monomeric cyclin D proteins would be rapidly degraded, leaving a constitutive activation of RB function and locking cells in a permanent G1-arrested state. Repression of p16 expression by pRB family proteins thus also constitutes a feedback loop to set up a balance between INK4-mediated inhibition and cyclin D-mediated activation of G1 progression. This function of p16, however, must be repressed in stem cells, which undergo continuous proliferation and self-renewal in vivo. We speculate that one mechanism to achieve this is through expression of BMI1 in the stem cell compartment.

Materials and methods

Cell culture, Western analysis, and antibodies

MEFs carrying mutant combinations in RB family genes, including RB1−/−; p107−/−; p130−/− (TKO) MEFs were previously described [Sage et al. 2000], and early-passage WEBS and SV40-transformed VA13 cells were purchased from American Type Culture Collection (ATCC). MEF, WEBS, VA13, and 293 cells were cultured in DMEM supplemented with 10% FBS, and Saos-2 cells were cultured in McCoy’s 5A supplemented with 15% FBS. Cells were lysed with RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 1 mM Na3VO4, 1 mM DTT, 1 mM PMSF, and a cocktail of protease inhibitors containing 25 mM leupeptin, 25 mM aprotinin, 150 mM benzamidine, and 10 mg/mL trypsin inhibitor). Antibodies to BMI1 [F6, Upstate Biotechnology], p16 (Ab-4, Neomarkers), human pRB [Ab-1; Neomarkers], mouse pRB (G3-245; BD Biosciences), p107 (C-18, Santa Cruz Biotechnology), p130 (Rb2; BD Biosciences), and actin (C-11, Santa Cruz Biotechnology) were purchased commercially.

Q-RT-PCR

Total RNA was extracted by RNeasy [Qiagen], and 1 µg was used for cDNA synthesis primed with Oligo(dT)20 primers (Invitrogen). The PCR protocol comprised incubations with nuclear lysis buffer (20 mM HEPES at pH 7.9, 0.5% NP-40, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dNTP, and protease inhibitor cocktail). After centrifugation at 4000 rpm for 5 min, the cell pellets were lysed by sonication on ice with nuclear lysis buffer (20 mM HEPES at pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and protease inhibitor cocktail). After centrifugation at 13,000 rpm for 10 min in a cold room, the lysates were diluted with an equal volume of dilution buffer (1% Triton X-100, 2 mM EDTA, 50 mM NaCl, 20 mM Tris-HCl at pH 7.9, and protease inhibitor cocktail). Immunoprecipitation was performed with an antibody specific to BMI1 [F6, Upstate Biotechnology], RING2, SUZ12, 3h-H3K27 [Cao et al. 2005], and normal mouse IgG as a control for 6 h or overnight at 4°C. After immunoprecipitation, 20 µL of salmon sperm DNA/protein G agarose [Upstate Biotechnology] were added and incubated for 1 h. Precipitates were sequentially washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl at pH 8.1), and TSE III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1), and washed twice with TE buffer before being eluted with 1% SDS and 0.1 M NaHCO3. The eluates were incubated for at least 6 h at 65°C to reverse the formaldehyde cross-linking. DNA fragments were purified by using the PCR Purification Kit [Qiagen]. PCR was performed using Platinum Taq polymerase [Invitrogen] and the following pairs of primers: mp16 exon 1a, 5′-CGAATCTGGAGAGACCCATC-3′ and 5′-ACATCTTCCGTCCTAA-3′; mp16 exon 2b, 5′-TCACCTAGACCTCTTCC-3′ and 5′-CAGCGAGACCGAAATATCCG-3′; mp16 exon 2c, 5′-GACAGTGATCTTCTTAA-3′ and 5′-CTCAACGCACACTCCTG-3′; and mp16 exon 2d, 5′-GACGAGATCTTCGCTAC-3′ and 5′-CCAGTGAGCTGACTG-3′.

ChIP assay

W188 or MEF cells [5 × 106] were treated with 1% formaldehyde for 10 min at room temperature, then 0.125 M glycine was added and incubated for 5 min at room temperature. The cells were lysed with cell lysis buffer on ice (10 mM HEPES at pH 7.9, 0.5% NP-40, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM dNTP, and protease inhibitor cocktail). After centrifugation at 4000 rpm for 5 min, the cell pellets were lysed by sonication on ice with nuclear lysis buffer (20 mM HEPES at pH 7.9, 25% glycerol, 0.5% NP-40, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, and protease inhibitor cocktail). After centrifugation at 13,000 rpm for 10 min in a cold room, the lysates were diluted with an equal volume of dilution buffer (1% Triton X-100, 2 mM EDTA), 50 mM NaCl, 20 mM Tris-HCl at pH 7.9, and protease inhibitor cocktail). Immunoprecipitation was performed with an antibody specific to BMI1 [F6, Upstate Biotechnology], RING2, SUZ12, 3h-H3K27 [Cao et al. 2005], and normal mouse IgG as a control for 6 h or overnight at 4°C. After immunoprecipitation, 20 µL of salmon sperm DNA/protein G agarose [Upstate Biotechnology] were added and incubated for 1 h. Precipitates were sequentially washed with TSE I (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl at pH 8.1), TSE II (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl, 20 mM Tris-HCl at pH 8.1), and TSE III (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl at pH 8.1), and washed twice with TE buffer before being eluted with 1% SDS and 0.1 M NaHCO3. The eluates were incubated for at least 6 h at 65°C to reverse the formaldehyde cross-linking. DNA fragments were purified by using the PCR Purification Kit [Qiagen]. PCR was performed using Platinum Taq polymerase [Invitrogen] and the following pairs of primers: mp16 exon 1a, 5′-CGAATCTGGAGAGACCCATC-3′ and 5′-ACATCTTCCGTCCTAA-3′; mp16 exon 2b, 5′-TCACCTAGACCTCTTCC-3′ and 5′-CAGCGAGACCGAAATATCCG-3′; mp16 exon 2c, 5′-GACAGTGATCTTCTTAA-3′ and 5′-CTCAACGCACACTCCTG-3′; and mp16 exon 2d, 5′-GACGAGATCTTCGCTAC-3′ and 5′-CCAGTGAGCTGACTG-3′.
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