Defective nucleolar localization and dominant interfering properties of a parafibromin L95P missense mutant causing the hyperparathyroidism–jaw tumor syndrome

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Abstract

The hyperparathyroidism–jaw tumor syndrome (HPT–JT) is a familial cancer syndrome that can result from germline inactivation of HRPT2/CDC73, a putative tumor suppressor gene that encodes parafibromin, a component of the transcriptional regulatory PAF1 complex with homology to the yeast protein Cdc73p. The vast majority of HRPT2/CDC73 germline mutations identified have been truncation or frameshift mutations, and loss of function due to missense mutation is rare. We report here a kindred with HPT–JT due to a germline L95P missense mutation in parafibromin. The mutant parafibromin was studied in vitro to understand the basis of its presumed loss-of-function. When transfected in cultured cells, the L95P mutant was expressed to a lower level than wild-type (wt) parafibromin, a difference that was not overcome by inhibition of the proteasomal degradation pathway. The L95P mutant parafibromin retained the ability to assemble with endogenous PAF1 complex components as evidenced by co-immunoprecipitation. Analysis of subcellular localization showed that the L95P mutant was markedly deficient in nucleolar localization compared to the wt, an impairment likely resulting from disruption of a putative nucleolar localization signal immediately upstream of the L95P mutation. Transfection of the L95P parafibromin mutant, but not the wt, enhanced cell cycle progression and increased cell survival in NIH-3T3 and HEK 293 cells, resulting apparently from dominant interference with endogenous parafibromin action. The simultaneous loss of nucleolar localization and acquisition of a growth stimulatory phenotype with the L95P mutation raise the possibility that parafibromin must interact with targets in the nucleolus to fully execute its tumor suppressor functions.

Introduction

mutation has also been reported in a minority of kindreds with familial isolated HPT (FIHP; Carpten et al. 2002, Howell et al. 2003, Simonds et al. 2004, Villablanc et al. 2004, Bradley et al. 2006, Guarnieri et al. 2006, Kelly et al. 2006, Mizusawa et al. 2006) and in up to 30% of patients with apparently sporadic parathyroid cancer (Shattuck et al. 2003, Cetani et al. 2004). HRPT2/CDC73 encodes parafibromin, a 531-amino acid putative tumor suppressor protein. The C-terminal region of parafibromin demonstrates sequence homology to Cdc73p, a budding yeast protein. Just as Cdc73p associates with the RNA polymerase II-associated PAF1 complex in yeast (Shi et al. 1997, Chang et al. 1999), mammalian parafibromin interacts with RNA polymerase II via a PAF1 complex, whose other protein components include Paf1, CTR9, Leo1 (Rozenblatt-Rosen et al. 2005, Yart et al. 2005, Zhu et al. 2005), and the WD40-repeat protein Ski8 (Zhu et al. 2005).

The vast majority of clinically identified HRPT2/CDC73 germline loss-of-function mutations reported to date have been truncation or frameshift mutations. In the present study, we describe a kindred with HPT–JT due to a germline L95P missense mutation in parafibromin. The mutant parafibromin was studied in vitro to better understand the basis of its impaired function. We report here that the L95P mutant retained the ability to interact with PAF1 complex components, but was expressed at a lower level than wild-type (wt) parafibromin and was deficient in nucleolar targeting. Furthermore, transfection of the parafibromin L95P missense mutant promoted cell survival and enhanced cell cycle progression, properties presumed to reflect dominant interference with tumor suppressor functions of endogenous parafibromin. These results suggest that nucleolar localization might be a sine qua non for the full anti-proliferative activity of parafibromin.

Case reports
Index case

The index patient (III-1, Fig. 1A) had incidental hypercalcemia discovered by routine blood testing at age 25 and primary HPT documented with an elevated intact parathyroid hormone (PTH) value of 135 pg/ml (normal 7–82) with a concurrent serum calcium of 11.1 mg/dl (normal 8.6–10.0) and hypercalciuria with 537 mg urinary calcium/24 h (normal 100–300). At cervical exploration, an enlarged right inferior parathyroid adenoma (1.2 cm diameter) was excised that was hypercellular on pathologic examination, and right and left superior parathyroid glands were biopsied that were normocellular. Post-operatively, the patient was rendered normocalcemic and has remained so for 3 years of follow-up. At age 18 and again at age 25, the patient had JTs removed by an oral surgeon with no operative or pathological reports available.

Other cases

The proband’s younger sister (III-2) is normocalcemic, rendered normocalcemic and has remained so for 3 years of follow-up. At age 18 and again at age 25, the patient had JTs removed by an oral surgeon with no operative or pathological reports available.
ureter associated with hypercalcemia (14 mg/dl), hypophosphatemia (1.9 mg/dl), and elevated PTH (204 pg/ml, normal 18–120). An enlarged cystic left inferior parathyroid adenoma (1.5 cm diameter) was excised at cervical exploration with no other parathyroid tissue identified. The patient’s calcium and phosphorus normalized postoperatively. The patient has remained normocalcemic during 16 years of follow-up, although he has experienced recurrent nephrolithiasis for the last 3 years. He has not had JTs. The proband’s aunt (II-2) had hypercalcemia discovered on an annual screening blood test at the age of 42, and she underwent cervical exploration for primary HPT, at which time, enlarged left and right inferior parathyroid adenomas (both 1.5 cm diameter) were excised. The left upper gland was identified and proven normal on biopsy. The patient has since remained eucalcemic for 11 years. She has not had JTs. The proband’s uncle (II-5) and grandfather (I-1) have also required surgery for primary HPT, but operative and pathology records are not currently available. None has reported JTs.

Materials and methods

HRPT2 mutational analysis

Patient genomic DNA was extracted from whole blood samples collected in EDTA tubes with informed consent approved by the NIDDK institutional review board and analyzed for HRPT2 mutation by GeneDx (Gaithersburg, MD, USA).

Mammalian cDNA expression constructs

Expression constructs containing cDNAs encoding AU5 epitope-tagged human parafibromin in pcDNA3 (Invitrogen; Woodard et al. 2005), and enhanced green fluorescent protein (GFP) fused in-frame at its C-terminus with human parafibromin in pEGFP-C1 (Clontech; Lin et al. 2007), were previously described. The corresponding L95P missense mutant cDNAs were prepared using the QuickChange II Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA). The coding region of the mutant cDNAs was confirmed by DNA sequencing.

Antibodies

Antibodies used for immunoblotting and/or immunoprecipitation were rabbit anti-human parafibromin antibody GRAPE (Lin et al. 2007), mouse anti-AU5 monoclonal (MMS-135R, Covance Research Products, Denver, PA, USA), mouse anti-β-actin monoclonal (A5316, Sigma), rabbit polyclonal anti-Leo1 (A300-175A, Bethyl Laboratories, Inc., Montgomery, TX, USA), rabbit polyclonal anti-Paf1 (A300-172A, Bethyl Labs), and mouse monoclonal anti-fibrillarin antibody (ab18380, Abcam, Cambridge, MA, USA). Secondary antibodies utilized in immunoblots or immunocytofluorescence were Cy3-conjugated donkey anti-mouse IgG (715-165-150, Jackson ImmunoResearch Labs, West Grove, PA, USA) and IR (infrared-dye labelled) secondary antibodies (anti-rabbit IR 800 and anti-mouse Red and Green) from LI-COR Bioscience (Lincoln, NE, USA).

Cell culture

Human cervical cancer-derived HeLa cells (Cat. no. CCL-2, ATCC, Manassas, VA, USA), human embryonic kidney HEK 293 cells (Cat. no. CRL-1573, ATCC), and mouse NIH-3T3 cells (Cat. no. CRL-1658, ATCC) were grown in 75 cm² flasks in DMEM supplemented with 10% fetal bovine serum (FBS), 4 mM L-glutamine, and penicillin/streptomycin at 37 °C and 5% CO₂. Empty vector or expression plasmids were transfected using Lipofectamine 2000 (Invitrogen) or PolyJet transfection reagent (SL 100688, SignaGen Laboratories, Ijamsville, MD, USA). The expression of the transfected proteins was verified by immunoblotting and/or immunofluorescence.

Immunoprecipitation

Immunoprecipitation with anti-AU5 antibody was performed according to the manufacturer’s instructions (Immunoprecipitation Starter Pack, Cat. no. 309410, Amersham Biosciences). Briefly, after transfection and 24 h incubation, HeLa cells were lysed with buffer A (250 mM NaCl, 50 mM Tris, pH 8.0, 5 mM EDTA, 0.5% NP-40, and 1× complete protease inhibitor cocktail (Cat. no. 1836170, Roche)). The lysate was centrifuged at 1500 g for 10 min. The supernatant was incubated overnight with AU5 antibody at 4 °C. The mixture was incubated with protein G Sepharose previously equilibrated in buffer A for 2 h at 4 °C. The beads were then washed three times with buffer A by centrifugation for 1 min, and the washes were discarded. Proteins were eluted from the beads with SDS sample buffer and detected by immunoblotting.

Gel electrophoresis, immunoblotting, chemiluminescence, and infrared imaging

Cell lysates were boiled with equal volume of Laemmli’s 2× gel loading buffer, and the hot solution was loaded onto 8% Tris–glycine SDS-PAGE gels (Invitrogen) to separate the proteins, followed by transfer of the proteins on to 0.45-μm nitrocellulose...
membrane. Membranes were blocked with Tris-buffered saline (TBS) or PBS (pH 7.4) containing 0.1% Tween 20 and 5% non-fat dry milk (blocking buffer), and incubated overnight with primary antibodies in the same buffer. The membranes were then washed seven times for 5 min each with the above buffer without milk, followed by a 1 h incubation in blocking buffer including IR-labeled secondary antibodies (dilution 1: 20 000) protected from light. Then, the membranes were washed four times and scanned for detecting the protein signals using the Odyssey infrared imaging system (LI-COR, Bioscience). For the quantification of the intensity of the protein bands, membranes were dually probed, with the β-actin signal used as a loading control.

Protein decay rate analysis

HeLa cells were transfected with empty pcDNA3 vector, or cDNAs encoding AU5-tagged wt or L95P mutant parafibromin. At 6 h after transfection, cells were treated with cycloheximide (Sigma) at a final concentration of 50 μg/ml. At the indicated time points after adding cycloheximide, cells were collected, and whole cell lysates were subjected to immunoblotting using GRAPE anti-parafibromin or anti-β-actin antibodies, with quantification by Odyssey infrared imaging as described above. Linear regression analysis of the natural logarithm of the relative actin-normalized parafibromin protein band intensity versus time was performed using the Linear Regression analysis program of Prism software version 5.0b for Macintosh OSX (GraphPad Software, San Diego, CA, USA).

Calculation of parafibromin half-life

Calculation of the parafibromin half-life was performed as previously described for c-myc (Lin et al. 2008). In brief, according to the linear regression analysis described above, the y-value at time = 0 for all plots was 4.61 (the natural log of 100(%) relative expression), and a y-value of 3.91 (indicating a relative expression of 50(%)) maps to the time corresponding to the parafibromin protein half-life. The Prism software program calculated the slope (m) ± S.E.M., y-intercept (b) ± S.E.M., r² value, and x-value at y = 3.91 (half-life) of the best-fit lines as part of the linear regression analysis. In order to calculate the S.E.M. of the half-life, the relation y = mx + b was first reordered as x = (y – (b ± S.E.M.))/m ± S.E.M., where x is the half-life, y = 3.91 (the natural log of 50(%)), b is the mean y-intercept, and m is the mean slope of the best-fit line. The S.E.M. of the half-life (S.E.M.,) under each condition was then estimated using the following equation, assuming b and m to be Gaussian variables: S.E.M., = ((3.91 – b)/m) × √((S.E.M.,)²/b² + (S.E.M.,)²/m²) (Motulsky 1995). Student’s unpaired t-test was used to evaluate for statistical significance.

Immunofluorescence

HeLa cells were transfected as described above and plated in chambers of poly-d-lysine-coated chamber slides and incubated for 6 h. The culture medium was discarded, and the cells were rinsed with PBS and fixed in 2% formaldehyde for 15 min. The slides were washed two times with PBS and incubated with PBS containing 8% FBS/azide (buffer A) for 5 min at room temperature. Then, primary antibody was diluted in buffer A containing 0.2% saponin (buffer B) and incubated at room temperature for 1 h. The slides were rinsed three times with buffer A and then two times with PBS, and the chamber was removed from the slides. Then, mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vectashield H-1200) was added, and the slides were incubated for 5 min in order to provide nuclear counterstaining. Finally, the slides were covered with a cover slip, sealed, and kept at 4 °C until the time of confocal laser scanning microscopy.

Laser confocal imaging

Images were acquired on a confocal microscope (Leica TCS-SP2 – Leica Microsystems GmbH, Mannheim, Germany) using a 60× oil immersion objective. Fluorescein was excited with an argon laser (Enterprise model 651, Coherent Inc., Santa Clara, CA, USA) at 488 nm, DAPI with u.v. light at 364 nm, and Cy3 cyanine dye with a yellow helium neon laser at 594 nm. DIC images were acquired simultaneously using a transmitted light detector. Dyes were acquired in separate channels to minimize crosstalk. Images were processed using Leica TCS-SP2 software, Imaris v6.2 (Bitplane AG, Zurich, Switzerland) and Adobe Photoshop CS3 (Adobe Systems).

Cell cycle analysis by flowcytometry and cell proliferation assay

For analyzing cell cycle distribution, NIH-3T3 and HEK 293 cells grown in 10 cm cell culture dishes were transfected with empty vector GFP vector as control, and either with GFP-wt or L95P parafibromin.
After 48 h of transfection, cells were treated with trypsin and harvested in PBS using brief centrifugation. Cells were then treated with Vindelov’s propidium iodide (PI) (TBS containing PI (50 μg/ml), RNase A (0.7 U/ml; Sigma Chemical Co.), and NP-40 (0.1%)), and were stored at 4 °C for at least 2 h in dark prior to the acquisition. Samples were then run on a LSRII flow cytometer (BD, San Jose, CA, USA), and 50,000 cells were acquired. The intensity of PI was analyzed in GFP-expressing cells using the cell cycle platform (Dean/ Jet/Fox mathematical model) of FlowJo software (Tree Star, Ashland, OR, USA).

Cell viability was measured in vitro using the MultiTox-Fluor Multiplex Cytotoxicity Assay kit (Promega). Briefly, cells were plated into the wells of a 96-well plate at 5000 per well in octuplicate. Cells were allowed to grow for 24 h and then transfected with pcDNA3 vector alone, or with cDNA encoding AU5-tagged wt or L95P mutant parafibromin. Forty-eight hours later, 20 μl/well of combined substrate solution of glycyl-phenylalanyl-amino-fluorocoumarin and bis-alanyl-alanyl-phenylalanyl-rhodamine were added. After incubation of 1 h at 37 °C in a humidified 5% CO2 atmosphere, the absorbance at 405 and 490 nM was recorded by using a microplate reader.

**Results**

**HPT–JT syndrome in a kindred associated with germline L95P missense mutation in parafibromin**

An index case with primary HPT and a history of JTs led to the identification of three generations of affected relatives that included four other individuals with primary HPT or menorrhagia associated with an abnormal endometrium as described in Case reports (Fig. 1A). The proband and three other affected individuals harbored a heterozygous T→C germline transition in exon 3 of the HRPT2/CDC73 gene that resulted in a change in codon 95 of parafibromin from leucine (CTT) to proline (CCT) (Fig. 1A). An identical somatic HRPT2/CDC73 gene mutation was previously identified in an atypical parathyroid tumor taken from an individual with FIHP (Bradley et al. 2006). The leucine residue at position 95 in human parafibromin is highly conserved, and is found in the corresponding position in parafibromin homologs from other species including fly and worm (Fig. 1B). The conserved leucine is immediately downstream of a putative nucleolar localization signal (NoLS; Hahn & Marsh 2007, Lin et al. 2007; Fig. 1B).

Expression and stability of the L95P parafibromin mutant

Since the vast majority of germline and somatic HRPT2/CDC73 mutations identified in patients with HPT–JT, FIHP, or parathyroid cancer have been truncation or frameshift mutations, we presumed that the missense mutation identified in the HPT–JT kindred described here would impair the function of parafibromin. To better understand the molecular basis of its presumed loss of function, AU5 epitope-tagged and GFP fused versions of human L95P parafibromin mutant were made and compared to the wt in cDNA transfection experiments in mammalian cell lines. When transfected into HeLa cells, both the GFP- and AU5-tagged versions of the L95P parafibromin mutant were expressed and had electrophoretic mobility similar to the corresponding wt protein as detected with anti-parafibromin antibodies (Fig. 2A).

Quantitative immunoblotting by infrared imaging with normalization to the actin level showed that the L95P mutant parafibromin was expressed only to a level of about 50% of the corresponding wt GFP fusion and to a level of about 30% of the corresponding AU5-tagged wt protein (Fig. 2A). Measurement of transcript levels of the transfected tagged parafibromin cDNAs by quantitative RT-PCR showed that levels of transcripts containing the L95P mutant were higher, not lower, than wt transcripts excluding missense or non-sense-mediated mRNA decay as a mechanism for reduced expression of the tagged parafibromin mutants (Supplementary Figure 1, see section on supplementary data given at the end of this article). Because steady-state levels of both transfected L95P parafibromin mutants were lower than the corresponding wt proteins and could not be explained by lower transcript levels, we hypothesized that the missense mutation might destabilize the protein and confer accelerated degradation kinetics.

We therefore studied the kinetics of protein disappearance in cycloheximide-treated HeLa cells previously transfected with vector alone, or with AU5-tagged wt or L95P mutant parafibromin by quantitative immunoblotting (Fig. 2B). The expression of parafibromin was examined at multiple time points between 0 and 100 min by quantitative analysis of the immunoblots by infrared imaging, and normalized by reference to the level of actin in each sample. The use of anti-parafibromin antibody in these experiments allowed the simultaneous monitoring of endogenous and transfected parafibromin (the latter migrating slightly slower on gel electrophoresis because of its epitope tag; Fig. 2B). The natural logarithm of the
actin-normalized parafibromin protein expression level (relative to \( t=0 \)) was plotted over time, and linear regression analysis was used to estimate the parafibromin protein half-life assuming first-order kinetics (Fig. 2C). The estimated half-life of endogenous parafibromin was greater than that of the AU5 epitope-tagged wt parafibromin construct, which was in turn greater than that of the L95P mutant parafibromin construct (Fig. 2C; endogenous parafibromin half-life, \( 520 \pm 256 \) min; AU5-wt parafibromin half-life, \( 53 \pm 17 \) min; AU5-L95P parafibromin half-life, \( 32 \pm 7 \) min), but the differences among the estimated half-lives did not achieve statistical significance (endog. versus wt, \( P=0.098 \); endog. versus L95P, \( P=0.086 \); wt versus L95P, \( P=0.28 \); Fig. 2C).

To pursue the question of possible mutant protein destabilization, we examined whether inhibition of the endogenous proteasomal degradation pathway might
increase steady-state levels of the missense parafibromin preferentially compared to the wt. This experiment was suggested by previous evidence that missense mutants of tumor suppressor proteins undergo accelerated degradation by the proteasomal pathway (Xu & Attisano 2000, Yaguchi et al. 2004). Treatment of transfected HeLa cells with the proteasome inhibitor lactacystin increased the expression of both endogenous and transfected AU5 epitope-tagged parafibromin constructs compared to control (Fig. 2D). However, analysis of the actin-normalized expression levels by quantitative immunoblotting revealed no preferential increase in the relative expression of the L95P parafibromin missense mutant compared to the wt (Fig. 2D). Similar results were seen with the proteasome inhibitor MG132 (not shown). Interestingly, in both control and lactacystin-treated cells, transfection of wt or L95P mutant parafibromin increased the steady-state level of endogenous parafibromin (Fig. 2D). The mechanism behind this phenomenon is not clear but might reflect competition for limited degradation capacity or else the existence of positive feedback circuits, leading to increased HRPT2 promoter activity and/or enhanced stabilization of endogenous parafibromin protein. This effect may be subtle because it was not always readily demonstrable (cf. immunoblots in Fig. 2A).

Interaction of the L95P parafibromin mutant with PAF1 complex components

Mammalian parafibromin interacts with RNA polymerase II via a PAF1 complex (Rozenblatt-Rosen et al. 2005, Yart et al. 2005, Zhu et al. 2005), a function initially reported for the parafibromin homolog Cdc73p in yeast (Shi et al. 1997). Because impaired ability to interact with the PAF1 complex might account for the presumed loss-of-function of the L95P missense mutant, we examined the ability of wt and mutant parafibromin proteins to interact with PAF1 complex components by immunoprecipitation (Fig. 3). Both transfected wt and L95P mutant parafibromin could be immunoprecipitated with AU5 monoclonal antibody to their epitope tag, and both proteins could co-immunoprecipitate the PAF1 complex components Paf1 and Leo1 (Fig. 3A). Analysis of the precipitates by quantitative immunoblotting, however, revealed no preferential deficiency of the L95P parafibromin missense mutant in this assay, and for both the wt and mutant proteins, the amount of Paf1 and Leo1 precipitated simply reflected the amount of epitope-tagged parafibromin protein in the AU5 immunoprecipitates (Fig. 3B).

Subcellular localization of the L95P parafibromin mutant

As noted above, the highly conserved leucine that is mutated to proline in affected individuals from the HPT–JT family described here is immediately downstream of a putative NoLS and secondary NLS in parafibromin (Hahn & Marsh 2007, Lin et al. 2007; Fig. 1B). To see whether the mutation might have an impact on parafibromin targeting, the subcellular localization of the GFP-tagged L95P parafibromin mutant was compared with that of the corresponding wt GFP fusion protein and GFP alone in transfected HeLa cells by laser confocal microscopy (Fig. 4). The cells were also treated with DAPI and anti-fibrillarin antibodies to delineate the nucleus and nucleolus respectively. Whereas GFP alone was distributed throughout the cytoplasm and nucleus (Fig. 4A), the wt parafibromin GFP fusion protein was localized to the cell nucleus with strong nucleolar concentration, as evidenced by co-localization with the anti-fibrillarin antibodies, as previously reported (Hahn & Marsh 2007; Fig. 4B). In contrast, the L95P parafibromin GFP fusion showed a striking lack of nucleolar localization.
even though the protein was clearly targeted to the cell nucleus (Fig. 4C and D). The change in codon 95 of parafibromin from leucine to proline seemed to be particularly deleterious to nucleolar localization, since GFP fusions with parafibromin mutants bearing more conservative amino acid substitutions, such as to isoleucine (L95I) or alanine (L95A), demonstrated nucleolar localization resembling the wt on laser confocal analysis (Fig. 4E and F). Co-transfection of an excess of L95P mutant parafibromin did not block the nucleolar localization of the wt parafibromin GFP fusion protein (Supplementary Figure 2, see section on supplementary data given at the end of this article).

**Effect of the L95P parafibromin mutant on cell cycle progression and cell viability**

It has been previously shown that knockdown of endogenous parafibromin or transfection of patient-derived parafibromin mutants promotes cell cycle progression and/or cellular proliferation (Yart et al. 2005, Zhang et al. 2006, Lin et al. 2008). We wondered whether the presumed loss-of-function of the L95P parafibromin mutant might exhibit a phenotype in this regard, and we therefore studied the cell cycle distribution of cells transfected with GFP alone or GFP-tagged wt or L95P parafibromin by flow cytometric analysis in GFP-expressing cells.

**Figure 4** The L95P parafibromin mutation blocks nucleolar but not nuclear localization. HeLa cells were cultured in chamber slides and transfected with GFP vector control (A) or else GFP fusions with wild-type (Pfb wt) (B) or L95P mutant parafibromin (Pfb L95P) (C). Cells were then treated with the DAPI nuclear stain, immunostained using anti-fibrillarin antibody as a nucleolar marker, and analyzed by confocal laser fluorescence microscopy. Phase contrast and overlay (merge) of GFP fluorescent signal with the nucleolar marker without or with the nuclear stain are also shown. (D) Quantitation of nucleoli per HeLa cell transfected with GFP fusions with wild-type (Pfb wt) or L95 mutant parafibromin (Pfb L95P); total nucleoli counted, if red on fibrillarin-only-stained images; GFP-positive nucleoli counted, if yellow on merging of GFP fluorescence and fibrillarin-stained images. Means that are shown are from pooling of two or three experiments per transfection, with 19–24 cells (nuclei) analyzed per experiment. ***, two-tailed \( P \) value \(<0.0001\) compared to total, employing Student’s unpaired \( t\)-test. (E and F) Confocal laser fluorescence microscopic images of HeLa cells transfected with GFP fusions with L95I mutant (Pfb L95I) (E) or L95A mutant parafibromin (Pfb L95A) (F), and analyzed as indicated, in (A–C).
Compared to control, there was a significant loss in G1 phase and gain in G2/M phase cells in L95P parafibromin-transfected NIH-3T3 and HEK 293 cells (Fig. 5A and B). No significant differences from control were seen in wt parafibromin-transfected cells in either cell type.

The effect of transfected wt and mutant parafibromin on cell viability was also studied in cultured cells (Fig. 5A and B). Compared to control, there was a significant loss in G1 phase and gain in G2/M phase cells in L95P parafibromin-transfected NIH-3T3 and HEK 293 cells (Fig. 5A and B). No significant differences from control were seen in wt parafibromin-transfected cells in either cell type.
(Fig. 5C). Transfection of the L95P parafibromin mutant, but not the wt, significantly increased the survival of both NIH-3T3 and HEK 293 cells in this assay (Fig. 5C). In fact, the cell viability of wt parafibromin-transfected NIH-3T3 cells was reduced; however, this effect did not reach statistical significance (Fig. 5C, left panel). This outcome is somewhat at odds, with previous results employing NIH-3T3 cells from our laboratory (Woodard et al. 2005) and others (Zhang et al. 2006) showing anti-proliferative effects in response to acute transfection of wt parafibromin. The basis for this apparent discrepancy is not known but may relate to the different proliferation assays employed with the NIH-3T3 cells (tetrazolium salt conversion colorimetric assay (Woodard et al. 2005) versus focus formation and soft agar colony formation assays (Zhang et al. 2006) versus dual fluorometric substrate cell viability assay (present study)).

**Discussion**

The identification of a rare missense mutation in a family with HPT–JT provided an opportunity to study the mutant parafibromin function in vitro to understand the basis of its presumed loss of tumor suppressor function. We observed that the ability to associate with PAF1 complex components Paf1 and Leo1 was preserved in the L95P parafibromin mutant, perhaps unsurprising since the point mutation is remote from the core Cdc73p homology domain encompassing residues 233–525 (Pfam ID PF05179; Finn et al. 2008) presumed to mediate the evolutionarily conserved interaction of the HRPT2/CDC73 gene product with the PAF1 complex (Shi et al. 1997, Chang et al. 1999). The key differences from wt parafibromin we observed in the L95P mutant were its lower expression level, its impaired nucleolar localization, and its ability upon transfection to stimulate cell cycle progression and enhance survival of cells expressing a normal complement of endogenous parafibromin.

The lower expression level of the L95P parafibromin mutant might certainly account in part for its presumed loss-of-function. The difference in expression levels between wt and L95P mutant parafibromin was not reduced or eliminated by treatment with proteasome inhibitors, however, suggesting that accelerated proteasomal destruction of the missense mutant was not occurring as has been described for other mutant tumor suppressor proteins (Xu & Attisano 2000, Yaguchi et al. 2004). Whether there is a causal relationship between the impaired nucleolar localization of the L95P parafibromin mutant, suspected to result from disruption of a nearby strong NoLS (Hahn & Marsh 2007), and its lower protein expression level and possibly decreased stability compared to the wt remains an open question.

Whatever the mechanistic basis for its low steady-state protein levels, the quantitative difference in expression from the wt only underscores the significance of the qualitative effects observed for the L95P parafibromin mutant. Despite its documented lower expression level compared to wt, the L95P missense mutant has a distinct pro-cell growth and survival phenotype in transfected cells: the mutant tumor suppressor protein enhanced cell cycle progression and increased survival of both NIH-3T3 and HEK 293 cells. The distinct phenotype shown by the parafibromin mutant argues strongly against the view that the L95P missense substitution is a null mutation that produces an inert and inactive protein. The demonstration of the growth and survival-promoting phenotype of the mutant parafibromin, which are qualities that are opposite to those expected of a tumor suppressor protein, instead supports the view that the L95P mutant is blocking or interfering with the functions of the endogenous parafibromin present in the transfected cells.

Dominant interfering mutants of cell regulatory molecules are those that retain some protein–protein interactions of the parent but lose others as a result of the mutation. In this formulation, the retained interactions of the L95P parafibromin mutant with the PAF1 complex components might fall into the former category, while the loss of nucleolar targeting might reflect lost protein–protein interactions falling into the latter grouping. The combination of lost nucleolar localization with acquisition of a growth stimulatory phenotype following a single amino acid substitution in the L95P mutant raises the intriguing possibility that parafibromin must interact with targets in the nucleolus to execute its full repertoire of tumor suppressor functions. Examining how the loss of nucleolar targeting and associated protein–protein interactions as a result of the L95P missense mutation could be directly linked to its growth and survival-promoting phenotype may provide significant insight into the pathogenesis of sporadic and HPT–JT-associated parathyroid cancer.

**Supplementary data**

This is linked to the online version of the paper at http://dx.doi.org/10.1677/ERC-09-0272.

**Declaration of interest**

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.
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References


