**Light Chain 1 of Microtubule-associated Protein 1B Can Negatively Regulate the Action of Pes1**

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Pes1 was first identified as the locus affected in the zebrafish mutant *pescadillo*, which exhibits severe defects in gut and liver development. It has since been demonstrated that loss of Pes1 expression in mammals and yeast affects ribosome biogenesis, resulting in a block in cell proliferation. Pes1 contains a BRCA1 C-terminal domain, a structural motif that has been shown to facilitate protein-protein interactions, suggesting that Pes1 has binding partners. We used a yeast two-hybrid screen to identify putative interacting proteins. We found that light chain 1 of the microtubule-associated protein 1B (Mtap1b-LC1) could partner with Pes1, and deletion analyses revealed a specific interaction of Mtap1b-LC1 with the Pes1 BRCA1 C-terminal domain. We confirmed the integrity of the interaction between Pes1 and Mtap1b-LC1 by co-immunoprecipitation experiments. Protein localization studies in NIH3T3 cells revealed that exogenously expressed Pes1 was typically restricted to nuclei and nucleoli. However, exogenous Pes1 was found predominantly in the cytoplasm in cells that were forced to express Mtap1b-LC1. We also observed that the expression of endogenous Pes1 protein was significantly reduced or undetectable in nuclei when Mtap1b-LC1 was overexpressed, implying that a dynamic interaction exists between the two proteins and that Mtap1b-LC1 has the potential to negatively impact Pes1 function. Finally, we demonstrated that, as is the case when Pes1 expression is depleted by shRNA, overexpression of Mtap1b-LC1 resulted in diminished proliferation of NIH3T3 cells, suggesting that Mtap1b-LC1 has the potential to repress cell proliferation by modulating the nucleolar levels of Pes1.

Ribosome biogenesis is a strictly regulated process that when disrupted has been shown to correlate with cancer and other human diseases (1). The mechanisms underlying ribosomal RNA (rRNA) processing and the assembly of pre-ribosomal subunits have been thoroughly described in both yeast and mammals (2). A combination of genetic and biochemical experiments has demonstrated that Pes1 is required for ribosome biogenesis in both cases (3, 4). Although originally identified as a mutant that affects embryonic development in the zebrafish, Pes1 is an evolutionarily conserved protein and is indispensable for viability of yeast and mice (3, 5–9). The analysis of Pes1−/− mouse embryos revealed an essential requirement for Pes1 during pre-implantation stages of development because blastomeres lacking Pes1 failed to proliferate and exhibited a dramatic reduction in the abundance of ribosomes (3). In addition, recent studies have implicated Pes1 during transcriptional control of gene expression and in cell immortalization (10, 11). Pes1 contains a BRCA1 C-terminal (BRCT)3 domain that is necessary for normal Pes1 function (7, 8). BRCT domains have been identified in a wide variety of proteins, and it has been proposed that this domain mediates protein-protein interactions (12, 13). The presence of a BRCT domain, therefore, suggests that Pes1 interacts with other proteins as part of its activity in controlling ribosome biogenesis and cell proliferation. Indeed, Pes1 has previously been isolated in large protein complexes that are associated with a variety of functions. These include interactions with upstream binding transcription factor (Ubtf/UBF1), RNA polymerase I, and a block of proliferation protein 1 (Bop1) (14, 15), which are involved in the synthesis and assembly of the 60 S ribosomal subunits as well as origin recognition complex 6 (ORC6), which is required for DNA replication (4), and regulator of ribosome biogenesis 1 (RBB1), which has roles in chromosome segregation and cell cycle progression (16–19).

To identify new interacting partners of Pes1, which may shed light on the role of Pes1 in controlling mammalian cell proliferation and ribosome biogenesis, we used full-length Pes1 and the Pes1 BRCT domain as bait in two independent yeast two-hybrid screens. Here we report the characterization of the Pes1 interaction with light chain 1 of microtubule-associated protein 1B (Mtap1b-LC1), which was found to interact with Pes1 in both screens and whose forced expression in NIH3T3 cells inhibited Pes1 action.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—NIH3T3 cells were cultured in high glucose Dulbecco’s modified Eagle’s medium (Sigma, D-5761) supplemented with 10% (v/v) Fetal bovine serum (HyClone), 0.2 mM L-glutamic acid, 1.0 mM sodium pyruvate, penicillin (100 units/ml), and streptomycin (100 μg/ml) in a 37 °C humidified incubator with a 5% CO2 atmosphere. HEK293T cells were cultured similarly but with 10% (v/v) fetal bovine serum (HyClone).

**Received for publication, November 29, 2006, and in revised form, February 15, 2007 Published, JBC Papers in Press, February 18, 2007, DOI 10.1074/jbc.M610977200**

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Pes1 Interacts with Mtap1b-LC1

Yeast Two-hybrid Assay—The MATCHMAKER GAL4 Two-Hybrid System 3 from BD Biosciences was used to identify interacting proteins for Pes1. Yeast media were obtained from BD Biosciences. Full-length mouse Pes1 cDNA (7) was cloned into the pGBK7 vector (BD Biosciences; Pes-GGBK7), resulting in a fusion of the DNA binding domain of the yeast GAL4 transcription factor (Pes-FL). In parallel, cDNA encoding the BRCT domain (bases 936–1239) was amplified from the full-length Pes1 plasmid using the oligonucleotides CCGGGATCCGGAGAAGAAGAG and CCCCTAGATGGG-GAGTACTCT. The PCR product was subcloned into pDNR-3 (BD Biosciences) and subsequently into pGBK7 (BRCT-pGBK7), resulting in the fusion of the BRCT domain (amino acids 312–414) with the GAL4 DNA binding domain (Pes-BRCT). Saccharomyces cerevisiae (strain AH109) was transformed with either the Pes-GGBK7 or the BRCT-pGBK7 plasmid and mated with a transformed mouse 11-day embryo MATCHMAKER cDNA Yeast Library (strain Y187, BD Biosciences). Expression of Pes-FL and Pes-BRCT did not have any toxicity or auto-activating effects in yeast. The resulting pool of diploid yeast was plated onto either low stringency (lacking adenine, histidine, leucine, and tryptophan) or high stringency (lacking adenine, cytosine, glycine, histidine, leucine, and tryptophan) selection plates and screened according to the manufacturer’s protocol. Yeast plasmid DNA was extracted from isolated colonies and used to transform Escherichia coli. DNA recovered from E. coli was sequenced and characterized using the BLAST data base (www.ncbi.nlm.nih.gov/BLAST). All plasmids isolated from E. coli that contained sequences encoding proteins or parts of proteins were tested for transcriptional auto-activation and retested for interaction with Pes1 in yeast.

Isolation of Mtap1b-LC1 cDNA and Expression of Mtap1b-LC1 Protein in Yeast and Mammalian Cells—Mouse Mtap1b-LC1 cDNA was amplified from an IMAGE clone (865865, ATCC 1122372) using PfuTurbo™ DNA polymerase (Invitrogen) and the oligonucleotides GCGGATCTCTCCATGTGGTGAACCAGAC and GCGGTGACCTACATGTTCTCTTGAGCCATGC. The purified PCR product was introduced into pACT2 (BD Biosciences) to generate Mtap1b-LC1-GAL4 fusion protein in Y187 yeast and into pcDNA6-HA (20) to express HA-tagged Mtap1b-LC1 protein under the control of the cytomegalovirus promoter (HA-Mtap1b-LC1). This plasmid also confers resistance to blasticidin S. Expression plasmids were introduced into HEK293T cells and NIH3T3 cells by transient transfection using Lipofectamine PLUS reagent (Invitrogen). For immunoprecipitation, 5 μg of total protein was pre-cleared with 1 μg of normal rabbit IgG and 40 μl of protein A-agarose (Sigma) slurry and incubated with 2 μg of either rabbit anti-HA (Santa Cruz), rabbit anti-c-Myc (Sigma), or rabbit anti-albumin (Accurate Chemical and Scientific Corp.) antibody. Immunoprecipitated proteins were separated by SDS-PAGE, transferred to Immoblot polyvinylidene difluoride membrane (Bio-Rad), probed with the appropriate antibodies, and detected using SuperSignal West Pico chemiluminescent substrate (Pierce). The antibodies used for immunoblotting included rabbit anti-C-Pes (1:1,000) (3), mouse anti-HA (Santa Cruz Biotechnology; 1:500), rabbit anti-HA (Santa Cruz Biotechnology), mouse anti-c-Myc (Sigma; 1:500), mouse anti-β-actin (Sigma; 1:4,000), rabbit anti-IgG-horseradish peroxidase (Bio-Rad; 1:7,000), and mouse anti-IgG-HRP (Bio-Rad; 1:6,000 or Sigma; 1:10,000).

Depletion of Pes1 by Lentiviruses Expressing shRNAs—The following complementary oligonucleotides (Integrated DNA Technologies) encoding two short hairpin RNAs (Pes1-A1 and Pes1-C1) were designed to knock down Pes1: Pes1-A1, TGTT-TCAATGTGAGACCCATTCTCAAGAGAATGTGCTCAGCATGACACTTTTTTGGGAG and TCGAGGTCTCTCTCATTGATATGTTTGACATCCTCATCCTTTTGGGAG and TCGAGTTTTCATTAATAGGTGTTTCGCGGAAAATCTTTTCTTAAATATGTGCATGACACA; Pes1-C1, TGGTGTAGTTTGGCAGGCGGAAATTCAGAAGTTCCGCTGCAACATCCTCTTTTGGGAC and TCGAGGTCTCTCTCATGATAGGTGTTTCGCGGAAAATCTTTTCTTAAATATGTGCATGACACA. The resulting plasmids were annealed and cloned into the lentiviral vector plasmid pLentiLox 3.7 (pL.L3.7), and the resulting plasmids were used to generate replication-defective lentiviruses using standard procedures (22). NIH3T3 cells were plated at 3 × 10^5 cells/well in a 6-well plate. Culture medium was removed 4 h after plating, and cells were infected with 0.5 ml of lentiviral supernatant overnight. Infection medium was aspirated and replaced with 300 μl of fresh viral supernatant in 300 μl of culture medium. After incubation for 8 h, infection medium was replaced with fresh culture medium
and incubated overnight, at which point the medium was supplemented with 8 μg/ml puromycin (Sigma) to select for infected cells.

**Cell Proliferation Assay**—For Pes1 depletion cell proliferation assays, 2 × 10^5 puromycin-resistant lentivirus-infected NIH3T3 cells were plated in triplicate in p60 culture dishes with puromycin-containing medium, and cells were counted using a hemocytometer at days 1, 2, 3, and 4 after plating. Alternatively, NIH3T3 cells transfected with either the HA-Mtap1b-LC1 or empty vector plasmid were incubated for 48 h in the presence of 50 μg/ml blasticidin S (Invitrogen). Cells were replated and incubated for 2, 3, 4, 5, and 6 days under constant selection with blasticidin S. For counting, cells were trypsinized, and 6–8 samples of each cell suspension were counted using a Neubauer hemocytometer. In each case, the procedure was repeated in three independent experiments, and the final data were analyzed using Student’s t test.

**Immunostaining**—Cells were processed as described previously (3) and incubated with anti-rabbit HA (Santa Cruz; 1:500), anti-mouse c-Myc (Sigma; 1:1000), anti-mouse nucleophosphin (Zymed Laboratories Inc. 1:20), or anti-phosphohistone-3 (Upstate; 1:1000). For cell counting, five random fields from each slide were analyzed with NIH ImageJ software. Data from three independent experiments were analyzed using χ² test.

**RESULTS**

**Depletion of Pes1 Inhibits the Proliferation of NIH3T3 Cells**—We have previously demonstrated that loss of Pes1 disrupts development of pre-implantation mouse embryos (3), and others have shown that mutations in Pes1 correlate with reduced proliferation of yeast cells (4, 8). Moreover, expression of Pes1 has been found in a wide variety of proliferating cells, including cultured cancer cells, and its expression is lost in non-dividing cells (8). Such studies imply that Pes1 is required for mammalian cell proliferation. To determine whether Pes1 was indeed required for the proliferation of cultured mammalian cells, we designed a series of shRNAs that were predicted to deplete Pes1 when expressed in NIH3T3 cells. These shRNAs were expressed from replication defective lentiviruses, which also conferred resistance to puromycin. We identified two shRNAs that could reduce levels of Pes1 as determined by immunoblot analyses of infected cell extracts (Fig. 1A). Pes1-A1 shRNA reduced Pes1 levels by >60%, whereas Pes1-C1 shRNA was more efficient and reduced levels of Pes1 by >86% relative to that found in cells infected with control virus. To test whether depletion of Pes1 affected cell proliferation, cells were infected with control lentivirus or lentivirus expressing either Pes1-A1 (vPes1-A1) or Pes1-C1 (vPes1-C1) shRNA and counted after growth in selective medium. Fig. 1B reveals that whereas control cells underwent a 25-fold expansion over 4 days in culture, cells expressing Pes1-A1 shRNA expanded by 14-fold, and cells expressing Pes1-C1 shRNA expanded by 5-fold. The level of apoptotic cell death was indistinguishable between control and Pes1-depleted cells (not shown), suggesting that the observed reduction in the expansion of Pes1-depleted cell numbers was most likely a consequence of reduced cell proliferation. To test this directly, proliferating cells were identified in control and Pes1-depleted cells using immunocytochemistry to detect phosphohistone H3, whose expression is restricted to cells in the G2/M phase of the cell cycle (Fig. 1C). The proliferation of NIH3T3 cells infected with vPes1-A1 was reduced by ~40% and that of vPes1-C1-infected cells by ~60% compared with cells infected with control virus. Similar results were obtained when S-phase incorporation of bromodeoxyuridine was measured (not shown). In sum, these data demonstrate that Pes1 is essential for the proliferation of mammalian cells in culture.

**Yeast Two-hybrid Analyses Identify Multiple Proteins That Potentially Interact with Pes1**—The confirmation that Pes1 is essential for mammalian cell proliferation allowed us to reason that Pes1-interacting factors could potentially represent a novel class of cell proliferation regulators. Pes1 contains a BRCT domain, which in other proteins has been shown to facilitate protein-protein interactions, possibly by interacting with phosphorylated regions of partners (12, 13, 23). The existence of such a potential interaction domain was consistent with our proposal that Pes1 binds to partners that are important for its function within the nucleolus and that, in doing so, mediates its activity. Fig. 2, panels A–C, arrows, shows that exogenously expressed Myc-tagged full-length Pes1 can be detected within the nucleolus of transfected cells, as described previously (3, 8). In contrast, when the BRCT domain was deleted from Pes1, the protein was found in the nucleus but was excluded from the nucleolus (Fig. 2, D–F, arrowheads). These data demonstrate that the BRCT domain is essential for the nucleolar localization of Pes1 and indicate a role for this domain in facilitating Pes1 function.

We next performed a yeast two-hybrid assay using either full-length Pes1 (amino acids 1–584) or the Pes1 BRCT domain (amino acids 312–414) from mouse as bait to detect potential Pes1 interacting proteins. We elected to include full-length...
Pes1 in case the BRCT domain required additional Pes1 sequences for efficient interactions. We screened a commercial pre-transformed yeast library (MATCHMAKER™, BD Biosciences) that contains the yeast GAL4 transcriptional activation domain fused to cDNA-encoded proteins derived from an E11.0 fetal mouse. This library was chosen because Pes1 mRNA was previously detected in mid-gestation mouse embryos by in situ hybridization (7). From the full-length Pes1 screen, we isolated 84 clones of which 42 contained partial sequences of Mtap1b, and 2 contained partial sequences of tubulin α1 (Tubα1; α-tubulin) (supplemental Table 1). The sequences of the other 40 clones did not represent open reading frames. From the BRCT domain screen we obtained 117 clones, 56 of which contained open reading frames (supplemental Table 1). The 56 candidate clones that were identified using the BRCT domain alone as bait encoded a diverse array of proteins. Importantly, one clone was identified that encoded Mtap1b, but none was found that encoded α-tubulin. Several other clones encoded proteins that have previously been shown to be present in the nucleolus. These nucleolar proteins included nucleolin (Ncl), nucleolar and coiled-body phosphoprotein 1 (Nolc1), Dis3/RRP44, exosome complex exonuclease RRP41 (Exosc4), DEAD/H (Asp-Glu-Ala-Asp/His) box polypeptide 27 (Ddx27), and DEAD-box protein 5 (Ddx5), which have been implicated in pre-rRNA processing and ribosome assembly (24, 25). Several other factors were identified that are involved in mRNA processing, including RNA binding region (RNPI, RRM) containing 2 (Rnpc2, Rbm39), THO complex subunit 2 (Thoc2), FIP1-like 1 (Fip1l1), thioredoxin-like 4A (Txnl4), Sin3-associated polypeptide p18 (Sap18), WW domain binding protein 5 (Wbp5), and splicing factor arginine/serine-rich 6 (Srs6). Other candidate interacting proteins identified in the BRCT domain screen have roles in cell cycle progression, including fusion, derived from (r12;16) malignant liposarcoma (Fus), melanoma-associated antigen family D1 (Maged1), and regulator of chromosome condensation 1 (Chc1/Rcc1). It is noteworthy that we also obtained three WD repeat-containing proteins, WD repeat domain 57 (U5 small nuclear ribonucleoprotein-specific) (Wdr57), histone cell cycle regulation-defective homolog A (Hira), and WD repeat and SOCS box-containing 1 (Wsb1), because it has been shown recently that Pes1 can interact with another WD repeat-containing protein, WD repeat domain 12 (Wdr12), which is required for ribosome biogenesis and cell proliferation (26).

Although the BRCT screen identified several interesting candidates, we chose to focus on Mtap1b because it was the only candidate common to both screens and, therefore, seemed most likely to represent a bona fide binding partner. To confirm that the Mtap1b “prey” fusion protein was responsible for the interaction with Pes1, yeast were transformed with either Mtap1b prey plasmid alone, with Pes1 bait plasmid alone, or with both plasmids simultaneously. Fig. 3A shows that growth of yeast could be supported in high stringency selection medium only when both prey and bait plasmids were present. Alignment of sequences obtained from 22 arbitrarily selected Mtap1b-prey plasmids with the published Mtap1b cDNA sequence revealed that three clones were identical to the Mtap1b nucleotide sequence encoding amino acids 2183–2431 (Fig. 3B; clone 1), and 19 clones contained the sequence encoding amino acids 2141–2359 (Fig. 3B; clone 2). The Mtap1b gene encodes a precursor protein that is processed to produce a heavy chain consisting of amino acids 1–2209 (HC) and a light chain (LC1) containing amino acids 2210–2464 (27). The observation that clone 1 is predicted to contain only amino acids that are present within light chain sequence allowed us to predict that Pes1 can interact specifically with light chain 1 of Mtap1b (Mtap1b-LC1).

**The BRCT Domain Is Sufficient for the Interaction between Pes1 with Mtap1b-LC1 in Yeast**—To identify the domains of Pes1 that are responsible for the interaction with Mtap1b-LC1, we generated bait plasmids encoding the GAL4 DNA binding domain fused to a series of carboxyl end-truncated forms of Pes1 (Fig. 4A). These fusion proteins were tested for their ability to interact with Mtap1b-LC1 again using the yeast two-hybrid assay, and we confirmed that each truncated form of Pes1 lacked intrinsic transcriptional activation activity (data not shown). Yeast cells were next co-transformed with bait plasmids expressing each carboxyl truncation of Pes1 along with Mtap1b-LC1. Fig. 4B shows that all truncations that included the Pes1 BRCT domain (Pes1-FL, Pes1-519, Pes1-492, and Pes1-414) as well as a fusion containing the Pes1 BRCT domain alone (Pes1-BRCT) grew in both low and high stringency medium. In contrast, a fusion protein that lacked the BRCT domain through a carboxyl end deletion grew only in low stringency medium and failed to grow under high stringency conditions. Cumulatively,
these data demonstrate that the BRCT domain is both necessary and sufficient to mediate the interaction between Pes1 and Mtap1b-LC1 when expressed in yeast.

**FIGURE 3.** Pes1 interacts with Mtap1b-LC1 by yeast two-hybrid analysis. A, confirmation of the interaction between full-length Pes1 (Pes1-FL) and Mtap1b-LC1 in yeast under low and high stringency growth conditions. Intrinsic DNA binding and transcriptional activation activity of Mtap1b-LC1 and Pes1-FL were tested via co-transformation with empty bait (pGBKT7) or prey (pGADT7) vectors, respectively. B, alignment of the nucleotide sequences encoding full-length Mtap1b, Mtap1b-LC1 (amino acids 2210–2464), and two independent cDNAs (clone 1, clone 2) identified by yeast two-hybrid analysis.

**FIGURE 4.** The Pes1 BRCT domain is responsible for the interaction of Pes1 with Mtap1b-LC1. A, schematic of full-length Pes1 and a series of Pes1 truncations used in yeast two-hybrid analyses showing the relative position of nuclear localization sequences (NLS, white boxes), the BRCT domain (gray box), acidic domains (black boxes), and a potential sumoylation site (Sumo, white oval). They include full-length Pes1 (Pes1-FL), the Pes1 BRCT domain (amino acids 312–414; Pes1-BRCT), and Pes1 truncations containing amino acids 1–519 (Pes1-519), 1–492 (Pes1-492), 1–414 (Pes1-414), and 1–320 (Pes1-320). B, yeast two-hybrid assay showing that only fusion constructs containing the Pes1 BRCT domain supported an interaction with Mtap1b-LC1 to facilitate growth in high stringency medium.

Co-expression of Pes1 and Mtap1b-LC1 in HEK293T Cells Facilitates an Interaction between the Two Proteins—Although we demonstrated that Pes1 and Mtap1b-LC1 interact in the yeast two-hybrid assay, the environment in mammalian cells is markedly different from that in yeast. We, therefore, exogenously expressed HA epitope-tagged Mtap1b-LC1 (HA-Mtab1b-LC1) along with Myc epitope-tagged Pes1 (Myc-Pes1) in HEK293T cells and asked whether the two proteins could be immunoprecipitated as a complex. Protein complexes containing HA-Mtap1b-LC1 were immunoprecipitated from transfected cell extracts using anti-HA antibodies, and precipitated protein complexes were subsequently subjected to immunoblot analyses using anti-Myc antibodies to detect Myc-Pes1. Fig. 5 shows that Myc-Pes1 could be detected in transfected cell extracts that had been subjected to immunoprecipitation with anti-HA antibody but not in extracts precipitated with an irrelevant antibody (anti-albumin) or mock-precipitated (no antibody), confirming that Pes1 and Mtap1b-LC1 can interact in cultured mammalian cells. In addition, a carboxyl end deletion of Pes1 that retained the BRCT domain (Pes1–414) could also be co-precipitated with HA-Mtap1b-LC1, as could the BRCT domain alone, although the efficiency through which the Pes1 BRCT domain alone could be precipitated with HA-Mtap1b-LC1 appeared to be less than that observed for the full-length Pes1 protein. In contrast, the Pes1–320 truncation that lacked 264 amino acids at the carboxyl end, which resulted in absence of the BRCT domain, could not be co-precipitated with HA-Mtap1b-LC1. To define whether the BRCT domain was essential for Pes1 interaction with Mtap1b-LC1, we generated a version of Pes1 that lacked only the BRCT domain (Myc-Pes1-delBRCT). Although this protein could be efficiently precipitated with anti-Myc antibodies, it could not be precipitated in conjunction with HA-Mtap1b-LC1 (Fig. 5). From these results...
we conclude that, as is the case in yeast, the BRCT domain is both essential and sufficient for the interaction of Pes1 with Mtap1b-LC1 in mammalian cells.

Expression of Mtap1b-LC1 Changes the Localization of Exogenous Pes1 from the Nucleus to the Cytoplasm—To define the localization of Myc-tagged Pes1 and HA-tagged Mtap1b-LC1, immunocytochemistry studies were performed on mammalian cells that had been transfected with appropriate expression plasmids. As expected, Myc-Pes1 was detected predominantly in nucleoli (Fig. 6, panels A–C, arrowheads) as reported previously (3, 8). However, Fig. 6, panels D–F, shows that exogenously expressed HA-Mtap1b-LC1 could be detected to be most abundant in the cytoplasm (arrows), and little if any staining was present in the nucleus and nucleoli of transfected cells. This localization is consistent with the detection of endogenous Mtap1b-LC1 in axons and perinuclear regions of neurons in primary culture (28). The finding that HA-Mtap1b-LC1 and Myc-Pes1 appeared to be most prominent in different cellular compartments was surprising, given that both proteins could be precipitated as a complex, so we asked whether the expression of both proteins together affected their localization within the cell. Rather dramatically, when cells were treated so as to express both HA-Mtap1b-LC1 and Myc-Pes1, Pes1 shifted from being predominantly nucleolar (Fig. 6, panels A–C) to being most abundant in the cytoplasm (Fig. 6, panels G–J). To quantify this dynamic change in the location of Myc-Pes1 in response to Mtap1b-LC1 expression, cells expressing Myc-Pes1 along with either enhanced green fluorescent protein (EGFP) or HA-Mtap1b-LC1 were scored for the presence of Myc-Pes1 in the nucleus or in the cytoplasm. Fig. 6K shows that Myc-Pes1 was found in the nucleus of 93% of cells that expressed both Myc-Pes1 and EGFP. In contrast, nuclear expres-
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Endogenous Pes1 Is Lost from the Nucleoli in Response to Overexpression of Mtap1b-LC1—Although the above result is consistent with a dynamic interaction between Pes1 and Mtap1b-LC1, it seemed formally possible that cytoplasmic co-localization could be a consequence of aggregates of inappropriately processed proteins being retained in the cytoplasm. However, if the interaction of Pes1 with Mtap1b-LC1 were genuine, we predicted that expression of Mtap1b-LC1 would influence the distribution of endogenous Pes1 within the cell. As shown in Fig. 7A, immunostaining experiments using anti-Pes1 antibodies confirmed that Pes1 is present primarily within the nucleus and is most abundant in nucleoli of NIH3T3 cells (panel A, arrows) (3, 8). Although Pes1 remained in the nucleus/nucleolus in cells that were forced to express EGFP (Fig. 7A, D–F, arrows), expression of HA-Mtap1b-LC1 resulted in the loss of Pes1 from the nucleus in the majority of transfected cells (Fig. 7A, A–C, arrowheads). A comparison between Mtap1b-LC1- and EGFP-overexpressing cells revealed that Pes1 was undetectable in the nucleus of 55% (p < 0.01; $\chi^2$ test) of Mtap1b-LC1-expressing cells (Fig. 7B) and that staining was generally found at a reduced level in the remaining 45% of cells. In contrast, the levels of Pes1 remained unchanged in 93% of cells expressing EGFP.

The loss of Pes1 within the nucleolus could also be explained by Mtap1b-LC1 affecting the stability of nucleolar structure, which is dependent upon the active synthesis of ribosomes. Therefore, to determine whether Mtap1b-LC1 expression in NIH3T3 cells disrupted the nucleoli, we investigated the localization of nucleophosmin (Npm1, B23), a characteristic nucleolar marker (29, 30), in cells forced to express either Mtap1b-LC1 or EGFP as a control. In both control and experimental cells, nucleophosmin was easily detected in the nucleolus (Fig. 7A, B–D, arrows) and that staining was indistinguishable between control cells and cells expressing Mtap1b-LC1 (Fig. 7B). From these data we propose that the change in localization and expression of Pes1 in response to Mtap1b-LC1 is a direct consequence of Pes1 interaction with Mtap1b-LC1.

Overexpression of Mtap1b-LC1 Leads to a Significant Reduction in Cell Proliferation in NIH3T3 Cells—The observation that expression of Mtap1b-LC1 results in loss of Pes1 in the nucleolus implies that Mtap1b-LC1 may negatively regulate the action of Pes1 and consequently affect cell proliferation. Our finding that Pes1 is required for NIH3T3 cell proliferation provided an assay by which we could test whether Mtap1b-LC1 could act as a negative regulator of Pes1 function. NIH3T3 cells were transfected with either HA-Mtap1b-LC1 plasmid or empty vector as control and cultured in medium supplemented with blasticidin S to select for cells containing the transfected plasmids. Fig. 8 shows that cells transfected with control plasmid underwent a 30-fold expansion over 6 days, whereas Mtap1b-LC1-expressing cells expanded 10-fold. From these
data, we conclude that Mtap1b-LC1 expression results in significantly curtailed proliferation of mammalian cells. Although we recognize that working with a system that requires exogenous expression of proteins in cultured cells does not always reflect a physiological situation, we believe our cumulative results suggest that Mtap1b-LC1 has an inherent capacity to control cell proliferation and ribosome biogenesis by modulating the nucleolar accumulation of Pes1.

**DISCUSSION**

Previous studies have demonstrated that Pes1 has an evolutionarily conserved role in ribosome biogenesis and is required for cell proliferation (3, 5–9). Here we show that the BRCT domain of Pes1 is essential for the nucleolar localization of the protein, suggesting that this domain mediates interactions between Pes1 and proteins that reside in the nucleolus and contribute to Pes1 action in controlling ribosome synthesis. Consistent with this proposal is the finding that mutations in the BRCT domain of the yeast homolog of Pes1 results in reduced cell cycle progression (8). Using yeast two-hybrid screens, we have identified a variety of candidate Pes1 BRCT domain-interacting proteins that are involved in nucleolar functions associated with ribosome biogenesis and cell cycle progression. Although our data confirm that the BRCT domain is important for Pes1 function, it is not the only region of Pes1 that is required for Pes1 activity. A transposon-mediated mutagenesis screen identified six Pes1 alleles that inhibited cell proliferation and rRNA processing (15). Importantly, none of these mutations mapped to the Pes1 BRCT domain. The majority of the alleles found to affect cell proliferation localized to a highly conserved block of amino acids close to the N terminus of Pes1, whereas others were found within the C-terminal end of the protein (15).

Although several Pes1-interacting partners were identified by our yeast two-hybrid analyses, somewhat surprisingly, other proteins that have been shown to interact with Pes1 previously, such as Bop1 (15), were undetected using either full-length Pes1 or the Pes1-BRCT domain as baits. This suggests that our yeast two-hybrid screens were not saturated and that additional Pes1 binding proteins are likely to exist. Although we believe that several of the candidate proteins that interacted with the Pes1-BRCT domain bait are likely to be bona fide partners of Pes1, we chose to focus our analysis on Mtap1b-LC1, because this was the only protein that was identified as a Pes1 partner in screens using both the full-length Pes1 and the Pes1-BRCT domain. Mtap1b mRNA encodes two peptides, a heavy chain (HC) and a light chain (LC1), which are derived by proteolytic processing, and we demonstrated that Pes1 interacts with the light chain portion of Mtap1b. Mtap1b has been shown to directly form cross-links between individual microtubules, stabilizing microtubule structure (31). In addition, Mtap1b can interact with other microtubule-associated proteins (32) as well as with actin stress fibers (27) and a number of non-microtubule-associated proteins that also regulate microtubule stability (33). Mtap1b has been best studied in developing and regenerating neurons, where it is expressed at high levels and is believed to be involved in neuronal morphogenesis, differentiation, and axon growth as well as to contribute to the maintenance of cytoskeletal integrity (34, 35). The role of Mtap1b in neurons also appears to have physiological significance because mice harboring a null allele of Mtap1b exhibit developmental defects in the central and peripheral nervous systems (36). Pes1 is also expressed in neurons (data not shown). Although any role for Pes1 in neurogenesis or neural activity has yet to be reported, given the co-expression of these two proteins in this cell type it seems possible that the neural phenotype seen in Mtap1b knock-out mice may in part be a consequence of Mtap1b-LC1 modulating the levels of nucleolar Pes1 in developing neurons.

Our finding that exogenous expression of Mtab1b-LC1 induces a loss of nucleolar localization of Pes1 supports the possibility that Mtap1b-LC1 acts as a Pes1 regulatory factor. In addition, we have demonstrated that cell proliferation is diminished in mouse NIH3T3 cells when either Mtap1b-LC1 is expressed or Pes1 expression is reduced using shRNA. Although it is possible that the reduction in cell proliferation observed in Mtap1b-LC1-expressing cells is unrelated to the concomitant loss of Pes1 from the nucleolus, we believe our cumulative findings support the proposal that Mtap1b-LC1 can act as a dominant negative regulator of Pes1 action. Although the mechanism through which Mtap1b-LC1 mediates depletion of Pes1 from the nucleoli remains unknown, the observation that these two proteins are binding partners and that Mtap1b-LC1 is predominantly localized to the cytoplasm suggests that Pes1 localization is dynamic and could involve shuttling between these two cellular compartments. The fact that Mtap1b-LC1 is able to interact with microtubules is also provocative, raising the possibility of a link between microtubule-mediated transport and cellular movement of Pes1. A number of studies have supported a model whereby microtubule-associated proteins mediate contacts between ribosomes and the microtubule network (37–39) that may facilitate the transport of ribosomes from their site of synthesis in the nucleolus (40–42). Additionally, other studies have demonstrated an association between ribosomes and microtubules during early stages of development (43) and even with the mitotic spindle during mitosis (44–46). No direct evidence has demonstrated that Pes1 interacts with any cytoskeletal components. However, confocal microscopy studies have revealed that Pes1 may align with the mitotic spindle of dividing two-cell-stage embryos (3). This interaction of Pes1 with the mitotic spindle apparatus seems to be restricted to early pre-implantation embryos because at later developmental stages and in cultured cells Pes1 associates with the periphery of condensed chromosomes in dividing cells (3). Nevertheless, the possibility remains that Mtap1b-LC1 could mediate an interaction between Pes1 and the spindle apparatus, at least during early developmental stages. Although most studies of Mtap1b-LC1 have centered on its interaction with microtubules, several roles have been proposed for microtubule-associated proteins in processes that may be independent of microtubule function. For example, in neuroblastoma cells Tau-1 has been identified in the fibrillar component of nucleoli as well as in the nucleolar organizer regions, both places where ribosome biogenesis is initiated (40), and Mtap1b-LC1 has been shown to interact with proteins involved in diverse processes such as mRNA trafficking and cAMP signaling (47, 48). It is, therefore, plausible that the inter-
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action of Pes1 with Mtap1b-LC1 is unrelated to the binding of Mtabp1b-LC1 to microtubules and could reflect an as-yet unidentified role for Mtabp1b-LC1 in controlling ribosome formation.

In summary, we have identified an interaction between Pes1 and Mtabp1b-LC1 that results in loss of Pes1 from the nucleolus and a reduction in cell proliferation and we believe our data demonstrate that Mtabp1b-LC1 has the potential to act as a negative regulator of Pes1 activity that could have important roles in controlling cell proliferation and ribosome biogenesis.

**Acknowledgments**—We thank Paula Traktman and Michele Battle for critical reading of the manuscript.

**REFERENCES**

1. Ruggero, D., and Pandolfi, P. P. (2003) *Nat. Rev. Cancer* 3, 179–192
2. Venema, J., and Tollervey, D. (1999) *Annu. Rev. Genet.* 33, 261–311
3. Lerch-Gaggli, A., Haque, J., Li, J., Ning, G., Traktman, P., and Duncan, S. A. (2002) *J. Biol. Chem.* 277, 45347–45355
4. Du, Y. C., and Stillman, B. (2002) *Cell* 109, 835–848
5. Adams, C. C., Jakovljevic, J., Roman, J., Harnpicharnchai, P., and Woolford, J. L., Jr. (2002) *RNA* 8, 150–165
6. Allende, M. L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N., and Hopkins, N. (1996) *Genes Dev.* 10, 3141–3155
7. Haque, J., Bober, S., Li, J., and Duncan, S. A. (2000) *Genomics* 70, 201–210
8. Kinoshita, Y., Jarell, A. D., Flaman, J. M., Foltz, G., Schuster, J., Sopher, B. L., Irvin, D. K., Kanning, K., Kornblum, H. I., Nelson, P. S., Hieter, P., and Grummt, I., and Baserga, R. (2004) *Mol. Cell. Biol.* 24, 4656–4665
9. Oeffinger, M., Leung, A., Tollervey, D., and Lueng, A. (2002) *RNA* 8, 626–636
10. Maiorana, A., Tu, X., Cheng, G., and Baserga, R. (2004) *Oncogene* 23, 7116–7124
11. Sikorski, E. M., Uo, T., Morrison, R. S., and Agarwal, A. (2006) *J. Biol. Chem.* 281, 24423–24430
12. Caldecott, K. W., Tucker, J. D., Stanker, L. H., and Thompson, L. H. (1995) *Biochem. Biophys. Res. Commun.* 210, 226–232
13. Ljungquist, S., Kenne, K., Olsson, L., and Sandstrom, M. (1994) *Mutat. Res.* 314, 177–186
14. Prisco, M., Maiorana, A., Guerzoni, C., Calin, G., Calabretta, B., Voit, R., Grummt, I., and Baserga, R. (2004) *Mol. Cell. Biol.* 24, 5421–5433
15. Lapik, Y. R., Fernandes, C. J., Lau, L. F., and Pestov, D. G. (2004) *Mol. Cell* 15, 17–29
16. Sakumoto, N., Yamashita, H., Mukai, Y., Kaneko, Y., and Harashima, S. (2001) *Biochem. Biophys. Res. Commun.* 289, 608–615
17. Iouk, T. L., Aitchison, J. D., Maguire, S., and Wozniak, R. W. (2001) *Mol. Cell. Biol.* 21, 1260–1271
18. Killian, A., Le Meur, N., Sesboye, R., Bourguignon, J., Bougard, G., Gauteriot, J., Bastard, C., Frebourg, T., and Flaman, J. M. (2004) *Oncogene* 23, 8597–8602
19. Schaper, M., Fromont-Racine, M., Linder, P., de la Cruz, J., Namane, A., and Yaniv, M. (2001) *Curr. Biol.* 11, 1885–1890
20. Battle, M. A., Maher, V. M., and McCormick, J. J. (2003) *Biochemistry* 42, 7270–7282
21. Turner, D. L., and Weintraub, H. (1994) *Genes Dev.* 8, 1434–1447
22. Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopina, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., Scott, M. L., and Van Parajs, L. (2003) *Nat. Genet.* 33, 401–406
23. Yu, X., Chini, C. C., He, M., Mer, G., and Chen, J. (2003) *Science* 302, 639–642
24. Andersen, J. S., Lyon, C. E., Fox, A. H., Leung, A. K., Steen, H., Mann, M., and Lamond, A. I. (2002) *Curr. Biol.* 12, 1–11
25. Olson, M. O., Dundr, M., and Szebeni, A. (2000) *Trends Cell Biol.* 10, 189–196
26. Holzel, M., Rohrmoser, M., Schle, M., Grimm, T., Harasim, T., Malamoussi, A., Gruber-Eber, A., Kremmer, E., Hiddemann, W., Bornkamm, G. W., and Eick, D. (2005) *J. Cell Biol.* 170, 367–378
27. Togel, M., Wiche, G., and Propst, F. (1998) *J. Cell Biol.* 143, 695–707
28. Mei, X., Sweat, A. J., and Hammarrack, J. A. (2000) *Brain Res. Bull.* 53, 801–806
29. Chou, Y. H., and Yung, B. Y. (1995) *Biochem. Biophys. Res. Commun.* 217, 313–325
30. Spector, D. L., Ochs, R. L., and Busch, H. (1984) *Chromosoma (Berl.)* 90, 139–148
31. Sato-Yoshitake, R., Shiomura, Y., Miyasaka, H., and Hirokawa, N. (1989) *Neuron* 3, 229–238
32. Hammarrack, J. A., Obar, R. A., Hughes, S. M., and Vallee, R. B. (1991) *Neuron* 7, 129–139
33. Ding, J., Liu, J., Kowal, A. S., Nardine, T., Bhattacharya, P., Lee, A., and Yang, Y. (2002) *J. Cell Biol.* 158, 427–433
34. Hirokawa, N. (1994) *Curr. Opin. Cell Biol.* 6, 74–81
35. Tucker, R. P. (1990) *Brain Res. Brain Res. Rev.* 15, 101–120
36. Meixner, A., Haeverkamp, S., Wasse, H., Fuhrer, S., Thalhammer, J., Kropf, N., Bittner, R. E., Lassmann, H., Wiche, G., and Propst, F. (2000) *J. Cell Biol.* 151, 1169–1178
37. Suprenant, K. A., Temporo, L. B., and Hammer, L. E. (1989) *Cell Motil. Cytoskeleton* 14, 401–415
38. Suprenant, K. A., Dean, K., McKeel, J., and Hake, S. (1993) *J. Cell Sci.* 104, 445–450
39. Suprenant, K. A. (1993) *Cell Motil. Cytoskeleton* 25, 1–9
40. Loomis, P. A., Howard, T. H., Castleberry, R. P., and Binder, L. I. (1990) *Proc. Natl. Acad. Sci. U. S. A.* 87, 8422–8426
41. Papasozomenos, S. C., and Binder, L. I. (1987) *Cell Motil. Cytoskeleton* 8, 210–226
42. Jessus, C., Huchon, D., Friederich, E., Francon, J., and Ozon, R. (1984) *Cell Sci.* 14, 295–301
43. Hamill, D., Davis, J., Drawbridge, J., and Suprenant, K. A. (1994) *J. Cell Biol.* 127, 973–984
44. Goldman, R. D., and Rehnbun, L. I. (1969) *J. Cell Biol.* 4, 179–209
45. Hirokawa, N., Takemura, R., and Hisanaga, S. (1985) *J. Cell Biol.* 101, 1858–1870
46. Silver, R. B., Cole, R. D., and Cande, W. Z. (1980) *Cell* 19, 505–516
47. Borland, G., Gupta, M., Magiera, M. M., Rundell, C. J., Fuld, S., and Yamamoto, J. (2006) *Mol. Pharmacol.* 69, 374–384
48. Tretyakova, I., Zolotukhin, A. S., Tan, W., Bear, J., Propst, F., Ruthel, G., and Felber, B. K. (2005) *J. Biol. Chem.* 280, 31981–31990