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mrtl - a translation/localization regulatory protein encoded within the human *c-myc* locus and distributed throughout the endoplasmic and nucleoplasmic reticular network

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Abstract

mrtl (*myc*-related translation/localization regulatory factor) is a previously uncharacterized protein synthesized from the first open reading frame contained within the human *c-myc* P0 transcript, ~800 nucleotides upstream of the Myc coding sequence. The mrtl protein, 114 amino acids in length, is projected to contain an N-terminal transmembrane domain and a highly charged C-terminal interaction domain with homology to numerous RNA-binding proteins. Using monoclonal antibodies raised against the hydrophilic C-terminal domain, endogenous mrtl was visualized in human breast tumor cell lines and primary mammary epithelial cells at the nuclear envelope and contiguous endoplasmic/nucleoplasmic reticulum. mrtl colocalizes and coimmunoprecipitates with translation initiation factor eIF2 α and the 40S ribosomal protein RACK1, and appears capable of binding specifically to the *c-myc* RNA. Inducible ectopic overexpression of *wild-type* mrtl interferes with the function of endogenous mrtl, which results in loss of Myc from the nucleus. Furthermore, treatment of cells with a peptide derived from the C-terminal domain displaces endogenous mrtl and causes a dramatic reduction in total cellular Myc protein levels. Together with our previous work demonstrating complete loss of tumorigenicity in association with ectopic expression of the *c-myc* P0 5'-UTR (containing the mrtl coding sequence), these results suggest that mrtl may serve an important function in regulating Myc translation and localization to the nucleus, perhaps ultimately contributing to the role of the *c-myc* locus in oncogenesis.

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Keywords

mrtl; *c-myc*; ORF1; nucleoplasmic reticulum; translational regulation; nuclear localization; breast epithelial cells

The *c-myc* protooncogene plays a major role in the regulation of cell growth, proliferation, differentiation, and apoptosis [Spencer and Groudine, 1991; Zanet et al., 2005]. The c-Myc protein (Myc) is thought to function primarily in the nucleus as a transcription factor for each of the three RNA polymerases. Through activation or repression of pol II target genes [Boyd and Farnham, 1997; Dang et al., 2006; Grandori et al., 1996; O'Connell et al., 2003], the Myc heterodimer plays a critical role in the decision to enter the cell cycle from quiescence [Holzel et al., 2001]. By stimulating activity of pol I (rRNA synthesis) [Arabi et al., 2005; Grandori et al., 2005], pol III [Gomez-Roman et al., 2003], as well as production of many other components of the translational machinery (e.g. ribosomal proteins, translation initiation factors) [Frye et al., 2003], Myc has a major impact on the overall rate of protein synthesis in the cell [Shiio et al., 2002]. Indeed, homozygous *c-myc* knockout cells are characterized by reduction in the rates of RNA and protein synthesis and exhibit dramatically prolonged doubling times [Mateyak et al., 1997].

The *c-myc* protooncogene is also unequivocally involved in the development and progression of many human malignancies. Mechanisms such as gene amplification, overexpression, or chromosomal translocation of *c-myc* are common in these tumors [Tirkkonen et al., 1998]. In fact, amplification of *c-myc* was found to be obligatory for de novo transformation of normal human breast epithelial cells [Elenbaas et al., 2001]. In addition, a complex relationship exists between *c-myc* and stem cell status [Okita et al., 2007; Watt et al., 2006], and this may also be relevant to Myc's role in oncogenesis. Yet, our current understanding of *c-myc* regulation and function is not sufficient to fully explain its profound influence on cell phenotype.

The structural organization of the human *c-myc* locus is complex (Fig. 1). Multiple distinct isoforms of the c-Myc protein may be produced by use of alternative translation initiation codons. Myc2 (p64), the predominant gene product, is thought to be largely responsible for the oncogenic phenotype attributed to the *c-myc* locus [Marcu et al., 1992]. Myc1 (p67) appears to have more growth inhibitory or tumor suppressor properties, and the stoichiometric balance between p64 and p67 is quite important in determining cell behavior [Hann et al., 1994]. An even smaller isoform, MycS (46–48 kDa), is generated by translation initiation further downstream within exon 2 [Xiao et al., 1998], and retains the ability to promote cell proliferation in spite of loss of most of the transcription activation domain.

Further upstream from the Myc coding sequence, there are two additional open reading frames positioned within the “5'-untranslated” region of the P0 transcript [Spencer and Groudine, 1991], potentially encoding two novel proteins which are entirely devoid of homology with each other or with Myc (see Accession M13930, *c-myc* P0 mRNA). The more proximal of these, designated MycHex1, has been identified in human cells as a monomeric 32 kDa or dimeric 58 kDa protein [Gazin et al., 1986], and received recent attention with the discovery of an internal ribosomal entry site (IRES) dedicated solely to enhancing translation of this 188 amino acid protein [Nanbru et al., 2001]. Characterization of the hypothetical product of the more distal open reading frame, referred to simply as “ORF1” in the literature, has been limited to in vitro translation [Bentley and Groudine, 1986].

The hypothetical ORF1 protein, which we have designated mrtl (*myc*-related translation/localization regulatory factor), is schematically diagrammed in the lower section of Fig. 1. Full-length mrtl is 114 amino acids in length (calculated 12.5 kDa), arginine-rich (11 residues)

and relatively basic (pI 8.3). The N-terminal region is very hydrophobic, and is expected to serve as a single transmembrane domain. The hydrophobic region is punctuated by a series of charged amino acids (RSER) beginning at position 30. An internal initiation codon potentially provides for synthesis of a slightly smaller isoform of mrtl (mrtx, 98 amino acids, 10.8 kDa) which would lack a major portion of the transmembrane domain. There are two consensus myristoylation sites in the middle region of the protein, which could further facilitate membrane association. The C-terminal sequence contains multiple alternating positively and negatively charged residues and exhibits considerable homology to a number of RNA-binding proteins. This sequence is not known to represent an RNA-binding motif, but rather serves as an interaction domain in these other proteins [Cazalla et al., 2002]. There are four sites for potential serine phosphorylation (consensus substrates for PKC, casein kinase II, PKA, and PKG), two of which are embedded in the midst of the charged residues of the C-terminal domain.

We sought to determine whether this hypothetical protein existed naturally in human cells, and to test our hypothesis that mrtl might function in cis to regulate translation of the Myc coding sequence further downstream. We reasoned that its synthesis from the upstream region of the *c-myc* mRNA would inherently place the nascent mrtl protein in the immediate vicinity of the regulatory RNA sequences controlling the rate of Myc translation, providing an excellent opportunity for mrtl to influence Myc protein synthesis by interacting with the *c-myc* mRNA itself and/or the translation regulatory molecules assembled there. We found that both ectopically produced and endogenous mrtl are distributed most prominently at the nuclear envelope, and also positioned throughout the endoplasmic reticulum and nucleoplasmic reticular structures. We determined that mrtl is closely associated with components of the translational machinery, including eIF2 α , eIF4G, and the 40S ribosomal protein RACK1. Finally, we determined that mrtl is tightly associated with cellular nucleic acids, possibly capable of directly binding the *c-myc* mRNA, and that mrtl may ultimately regulate the level of Myc in the nucleus.

MATERIALS AND METHODS

Generation and characterization of mrtl-specific monoclonal antibodies

A synthetic 16-mer peptide (CQTVLLRRSSRERERV) representing the C-terminal sequence of mrtl (amino acids 99–114) was conjugated to KLH through the N-terminal cysteine. Mice were immunized with the peptide-KLH conjugate by bilateral axillary and inguinal subcutaneous injections administered at 2–4 day intervals for a total of five immunizations. Two days after final immunization, the draining lymph nodes were harvested, lymphocytes recovered, and PEG-mediated fusion performed to generate hybridomas [Straughn et al., 2004]. The protocol was approved by the institutional IACUC committee. Culture supernatants from the resulting hybridomas were first screened by ELISA for antibody reactivity to ovalbumin-conjugated or unconjugated peptide, and ELISA-positive samples were secondarily screened by western blot using both in vitro translated mrtl and whole cell lysate. Hybridomas testing positive by western were subjected to two rounds of subcloning with confirmatory ELISA and western screening to generate hybridoma cultures with stable antibody production. From a large panel of hybridomas screened, two monoclonal antibodies with a high degree of specificity for mrtl yet very distinctive antigen recognition properties were obtained. mAb102 (IgM) preferentially recognizes in vitro translated and ectopically expressed mrtl, while mAb131 (IgG1) preferentially recognizes endogenous mrtl.

Development of stable transfectants capable of inducible ectopic expression of wild-type mrtl

The full-length wild-type mrtl coding sequence (from 10 bp upstream of the initiation codon to 3 bp past the stop codon) was PCR amplified using primers 5'-

CAGTGTCTAGAAATACGTGGCAATGCGTTGCTGG-3' and 5'-ACGACAAGCTTACTCTCAAAACCCTCTCCCT-3', and cloned into the NheI and HindIII sites of pBI-L (ClonTech) using XbaI and HindIII sites which had been incorporated into the 5'-tails of the primers. The resulting construct enables firefly luciferase and mrtI to be produced concomitantly under control of the bidirectional tet-inducible promoter. The double start knockout (dsko) construct was generated by site directed mutagenesis to change both potential mrtI initiation codons to stop codons, so that no mrtI-related protein could be produced. The constructs were confirmed by direct sequencing.

T47D Tet-ON cells (Clon-Tech) were transfected with either the experimental (ORF1) or control (dsko, pBI-L) expression constructs along with the linear hygromycin marker via Lipofectamine 2000 (Invitrogen). Stable transfectants were selected and continuously maintained in Hygromycin B (200 µg/ml) and G418 (500 µg/ml), using only Tetracycline-tested fetal calf serum in the growth media. Multiple independent clones were isolated and screened for doxycycline-inducible firefly luciferase activity, then further analyzed by PCR and RT-PCR to confirm the integrity of the ectopic sequence. The definitive test for ectopic mrtI-expressing clones (ORF17, ORF33, ORF43) was an increase in immunofluorescent staining for mrtI (using mAb102) following treatment with doxycycline (1 µg/ml for 72 to 96 hours).

Immunofluorescent staining

Cells grown on chamber slides were fixed in 4% paraformaldehyde in PBS for 30 minutes and permeabilized in 0.2% Triton X-100 for 10 minutes at room temperature, or fixed and permeabilized with ice-cold 100% methanol at -20°C for 15 minutes. Following PBS/glycine wash, the cells were blocked with 10% goat serum in PBS for 45 minutes, then incubated with primary antibodies (1:100 for commercial antibodies) for 1 hour at room temperature, washed three times in PBS and then incubated for 50 minutes with highly cross-adsorbed γ 1 or μ -chain isotype-specific secondary antibodies conjugated to AlexaFluor594 or 488. (All secondary antibodies were prepared from goat, except for imaging of mrtI with eIF4G, where fetal calf serum block and donkey secondary antibodies were used). Following final washes, the cells were mounted using ProLong Gold (Invitrogen). Immunofluorescent staining performed in the absence of primary antibody was used as a negative control.

The following commercial antibodies were used: c-Myc rabbit polyclonal N-262 (Santa Cruz); RACK1 mouse monoclonal (clone 20) (BD/Transduction); Ribophorin II rabbit polyclonal (H-300) (Santa Cruz); eIF2 α rabbit polyclonal (FL-315) (Santa Cruz); eIF4G goat polyclonal (D-20) (Santa Cruz); AlexaFluor488-conjugated mouse anti-Ki-67 (BD); Alexa Fluor488-conjugated mouse anti-BrdU (BD). DiOC₆(3) (3,3'-dihexyloxycarbocyanine iodide) was from CalBiochem. DAPI (4'-6-Diamidino-2-phenylindole) was from Sigma.

Confocal laser scanning microscopy

Images were acquired on a Leica DM IRBE inverted epifluorescence/Nomarski microscope outfitted with Leica TCS NT SP1 Laser Confocal optics, using sequential UV, 488 nm, and 568 nm laser line excitation, tight bandpass emission windows, and 40X (NA 1.25) and 100X (NA 1.4) planapochromat objectives. PMT voltages, laser power, offset, and pinhole size were held constant for all samples within an experimental set, and 4–6 scans were obtained per image.

Sequence analysis

The mrtI primary amino acid sequence was analyzed by a number of algorithms, including Antigen, Novotny, HelixMem, BLAST, and PROSITE.

Western analysis

Hot lysates (total cellular protein) were prepared by rapidly harvesting the cells in a solution containing 4% SDS and 720 mM beta-mercaptoethanol which had been heated to 99°C. Cold lysates were prepared by incubating cells at 4°C in a solution containing 1% NP-40 and 250 mM NaCl (buffer L100, optimized for recovery of Myc heterodimers [Sommer et al., 1998]). Protease inhibitors (aprotinin, leupeptin, AEBSF) and a phosphatase inhibitor cocktail were included in all cold lysis protocols.

Equal aliquots of lysate were separated on 17.5% SDS-polyacrylamide gels and transferred to 0.2 µm nitrocellulose. After blocking in TBS-Tween plus 5% non-fat dry milk, the membranes were incubated with anti-mrtl monoclonal antibodies for 1.5 hours at room temperature followed by overnight at 4°C. Washing, secondary antibody incubation (HRPO-conjugated goat anti-mouse IgG or goat anti-mouse IgM), and ECL detection were accomplished using standard protocols.

Northwestern analysis

Samples were separated by 17.5% SDS/PAGE and transferred to 0.2µm nitrocellulose. The blot was washed in Tris-buffered saline, and then incubated in Renaturation/block solution (40 mM Tris-Cl, pH 7.4; 60 mM KCl; 1 mM MgCl₂; 10 mg/ml BSA; 6% v/v glycerol; 0.25 mM spermidine; 50 µg/ml yeast tRNA; 250 mM β-mercaptoethanol; 0.01% NP-40; and 75 µM each ATP and GTP) for 2 hr at room temperature and then overnight at 4 °C. The ³²P-radiolabeled RNA probe (in vitro synthesized *c-myc* 5'-untranslated RNA in sense orientation) was then added and the incubation continued for 2 hrs at room temperature with constant gentle agitation on a rotating platform. The blot was washed once for 10 min in Ren/block solution containing heparin (1 mg/ml), then twice more for 5 min each in Ren/block solution, and the result obtained by autoradiography.

Immunoprecipitation

To solubilize endogenous mrtl under conditions conducive to maintenance of existing protein-protein interactions, untransfected T47D and MCF-7 cells were harvested in RNP lysis buffer (10mM Tris 7.4, 100mM NaCl, 2.5mM MgCl₂, 0.5% Triton X-100, 14 mM beta-mercaptoethanol, supplemented with AEBSF, leupeptin, aprotinin, and phosphatase inhibitor cocktail), sonicated on ice (5 sec × 2, Branson), and the supernatant used as a source for coimmunoprecipitation experiments. Lysates were diluted 1:1 in wash/incubation buffer WIB (10 mM Tris-Cl, pH7.4; 100 mM NaCl; 2.5 mM MgCl₂; 0.05% NP-40), precleared with normal rabbit IgG or mouse IgM bound to protein A/G-Plus agarose or anti-IgM agarose respectively, then incubated for 2 hours at 4°C with eIF2α or RACK1 antibodies bound to protein-A/G agarose or anti-IgM agarose respectively. Micrococcal nuclease was included during incubations to eliminate indirect co-immunoprecipitation through RNA. Bound proteins were washed x 3 with cold WIB buffer, collected by centrifugation, separated by SDS-PAGE and immunoblotted with mAb131 to mrtl.

Cell culture

Human breast cell lines MCF-10A, T47D, MCF-7, BT-20, and MDA- MB-231 were obtained from ATCC. T47D Tet-ON was obtained from Clon-Tech and maintained in G418. Primary human mammary epithelial cells (HMECs) were obtained from Clonetics. All cells were maintained in a humidified 37°C, 5% CO₂ environment and propagated using standard culture techniques and the suppliers' recommended media.

RESULTS

Analysis of ectopic and endogenous mrtl by western blot

To facilitate our characterization of mrtl, double stable transfectants of the T47D human breast carcinoma cell line were established in which *wild-type* mrtl could be ectopically expressed in a tetracycline-inducible manner. In addition, two distinct monoclonal antibodies were raised using a synthetic peptide derived from the C-terminal domain, projected to be the most hydrophilic and most antigenic region of the protein.

Hot lysates and detergent extracts were prepared from control and experimental clones, with and without doxycycline induction, and were examined for mrtl content by western blot using each of the mrtl-specific monoclonal antibodies (Fig. 2). Although they were raised against the same peptide antigen, the two mrtl-specific antibodies exhibited very distinct yet complementary epitope recognition properties, which proved very beneficial in analysis of mrtl regulatory properties. mAb102 (IgM) preferentially recognizes *in vitro* translated and ectopically expressed mrtl (with few or no post-translational modifications), while mAb131 (IgG1) preferentially recognizes endogenous mrtl (post-translationally modified and structurally integrated in the cell). Endogenous mrtl migrates at ~15 kDa, and is detected in both the experimental and control clones by mAb131. Ectopic mrtl (~12.5 kDa) is detected by mAb102 in the experimental clones ORF17 and ORF33, but not in dsko8 (double start knockout in which both mrtl initiation codons are mutated) or in T47D Tet-ON (the parent cell line). Ectopic mrtl was present at a low level in experimental clones even in the absence of doxycycline, but dramatically increased upon doxycycline induction.

Notably, ectopic and endogenous mrtl exhibited a marked difference in sensitivity to detergent extraction. Whereas ectopic mrtl was readily recovered from cells by mild detergent (1% NP-40) cold lysis, endogenous mrtl was much more tightly integrated into the cell and quite resistant to detergent extraction. These distinctions in molecular mass, detergent sensitivity, and epitope recognition suggest that the ability of ectopically expressed mrtl to function in the same manner as endogenous mrtl may be restricted, perhaps due to inherent limitations of a modifying enzyme (e.g. kinase) or structural interaction partner.

These results provided the first experimental evidence for the natural existence of the mrtl protein in breast epithelial (or any other human) cells. Some of the properties of endogenous and ectopic mrtl are compared and contrasted in Table 1. The functional differences between endogenous and ectopic mrtl are explored further in the data presented below.

Intracellular localization of ectopic mrtl

Next, we analyzed ectopic mrtl expression by indirect immunofluorescence (Fig. 3). Following induction with doxycycline, a dramatic increase in mAb102 reactivity was observed. This ectopically expressed mrtl was visualized predominantly at the nuclear envelope, along with a reticular cytoplasmic signal which was typically more intense nearer the nucleus than at the cellular periphery. This pattern is consistent with insertion of mrtl in the nuclear membrane and contiguous endoplasmic reticulum. In addition, many cells contained a single mrtl-positive spot near the center of the nucleus. Radial projections extending from the nuclear envelope inward were also occasionally seen.

This same staining pattern was observed in three independent experimental (mrtl-expressing) clones, and the same pattern was observed whether cells were fixed and permeabilized with paraformaldehyde/Triton X-100 or 100% methanol. A very low mAb102 signal was observed in experimental clones without doxycycline induction (leaky ectopic expression, consistent with western results). No signal was observed in control clones stably transfected either with

the parent vector with no mrt1 insert, or mutated mrt1 sequence in which both translation initiation codons were eliminated.

Relationship between ectopic mrt1 and the cell cycle

A marked incremental heterogeneity in ectopic mrt1 expression was consistently observed amongst the doxycycline-treated cells. We sought to determine the basis for this heterogeneity, and to rule out technical or artifactual elements that could contribute to this observation (Fig. 4). We determined that no limitation of doxycycline concentration, duration of doxycycline exposure, nor degree of permeabilization was responsible for the cell-to-cell variability in ectopic mrt1 expression. Likewise, this heterogeneity persisted, and in fact, no change in the mrt1 staining pattern was observed when cells were treated with proteasome inhibitors ALLN or lactacystin, indicating that targeted degradation of the mrt1 protein is not likely a major factor. Finally, to rule out mixed clonality of the mrt1-expressing clones, we seeded the cells at very low density, allowed sufficient time for proliferation of colonies from single cells, treated with doxycycline, and stained the cells for ectopic mrt1. Nearly all colonies contained both ectopic mrt1-positive and negative cells.

From these results, we concluded that the heterogeneity was inherent to ectopic mrt1 expression, and wondered whether cell cycle status might be a major determinant. We found that doxycycline induction of ectopic mrt1 expression tended to be associated with a decrease in the proportion of cells staining positive for Ki-67 (Fig. 4I–J). Furthermore, the mrt1-positive and Ki-67 positive cells tended to be mutually exclusive (the same was true for ectopic mrt1 and BrdU positive cells, Fig. 4K). Since Ki-67 stains all phases of the cell cycle except G₀, these results suggest either that ectopic mrt1 may only be allowed to accumulate in cells which have exited the cell cycle, or alternatively, that ectopic overexpression of mrt1 may actually drive cells into quiescence. A definitive cause and effect relationship has been difficult to establish, due in part to the very low rate of proliferation of these cells even without doxycycline induction (requiring in some cases >1 month for a single passage). Yet, this slow growth phenotype and inverse relationship between mrt1 and Ki-67 would be consistent with a possible *c-myc*-regulatory function for mrt1, considering the critical role that Myc plays in the decision of the cell to enter the cell cycle.

Association of endogenous mrt1 with the translation machinery

Endogenous mrt1 was visualized by indirect immunofluorescence and confocal microscopy in untransfected human breast tumor cell lines using mAb131 (Fig. 5). The intracellular distribution of endogenous mrt1 was very similar to that which had been observed for ectopically expressed mrt1 (nuclear envelope, reticular cytoplasm, intranuclear “target” spot). Endogenous mrt1 colocalized precisely with the lipophilic dye DiOC₆ (Fig. 5A–C), indicating that essentially all of the mrt1 visualized is membrane-associated, including the intranuclear structures. The rough ER marker ribophorin II also exhibited a very similar staining pattern and colocalized well with cytoplasmic mrt1 (Fig. 5D–F). Given that the nuclear envelope and rough ER are both studded with ribosomes, these findings suggest that endogenous mrt1 may reside in close proximity to the translational machinery, which would fit with our original hypothesis that mrt1 might be involved in translational regulation. In fact, we found that mrt1 colocalizes extensively with translation initiation factors eIF4G (Fig. 5G–I), eIF2 α (not shown), as well as the integral 40S ribosomal protein RACK1 (Fig. 5J–X). Furthermore, we found that endogenous mrt1 co-immunoprecipitates with both eIF2 α and RACK1 (Fig. 5Y), providing additional evidence for a close association of mrt1 with the translation machinery.

Possible direct interaction of mrt1 with the *c-myc* mRNA

BLAST searches using just the mrt1 C-terminal domain as the query sequence returned similar sequence elements in a number of RNA-binding proteins, including U1 snRNP-70kDa, RNA

helicase, U2AF, RNase III, and *Drosophila* Transformer-2. This sequence is not a recognized RNA-binding motif, but rather appears to represent a protein-protein interaction domain in these RNA-binding proteins [Cazalla et al., 2002], suggesting that mrtl might be part of a complex which regulates the translation, localization, or processing of mRNA. Yet, considering the overall arginine-rich nature of the mrtl sequence, its basic pI (calculated 8.3), and its inherent proximity to the *c-myc* mRNA (from which it is translated), we speculated that mrtl might be capable of directly interacting with the *c-myc* mRNA itself.

Northwestern analysis was used to assess the potential of mrtl to bind the *c-myc* mRNA (Fig. 6A). Cellular lysates obtained by a sequential biochemical fractionation protocol were separated by SDS/PAGE, transferred to nitrocellulose, and allowed to renature in a physiological buffer. Using the 5'-untranslated region of the *c-myc* mRNA as probe, we detected an RNA-binding protein interacting specifically with the *c-myc* mRNA which comigrated and cofractionated with mrtl, as indicated by western analysis performed on an identical blot.

This biochemical fractionation experiment also helped us to further characterize the relationship of endogenous mrtl to cell structure. The results indicate that nuclease digestion followed by high salt extraction of lysed cells is required to recover mrtl from the cell. Notably, high salt extraction alone is not effective in recovery of endogenous mrtl (data not shown), and Triton X-100 (1%) together with sodium deoxycholate (0.5%) did not facilitate additional recovery of mrtl from the cells either (consistent with the resistance of endogenous mrtl to detergent extraction noted in Fig. 2). Even following nuclease digestion, high salt extraction, and Triton/DOC, a substantial proportion (~30%) of mrtl remained in the insoluble pellet. The resistance of endogenous mrtl to detergent extraction suggests that intermolecular interactions beyond membrane insertion must be important for mrtl's structural integration in the cell.

To further investigate this possibility, we also performed *in situ* biochemical fractionation followed by immunofluorescent detection of mrtl. We found that nuclease digestion of cells prior to antibody incubation dramatically enhanced mrtl immunoreactivity, particularly within the nucleus (Fig. 6B). This apparent unmasking of the C-terminal epitope upon nuclease digestion supports a close association between endogenous mrtl and cellular nucleic acids (mRNA and perhaps chromatin), and further supports a possible role for mrtl in the integration of structural (e.g. membrane) and functional (e.g. translation) elements in the cell.

Endogenous mrtl in primary human mammary epithelial cells: detailed imaging of the nucleoplasmic reticulum and its central cisternal reservoir

To this point, our assessment of endogenous mrtl had been limited to immortalized breast cell lines. We felt it was important to examine mrtl in normal cells as well. For this purpose, primary human mammary epithelial cells (HMECs), derived from reduction mammoplasty and obtained through Clonetics (Lonza), were cultured briefly on multiwell slides and imaged for endogenous mrtl using mAb131. Unexpectedly, we found that the ER membrane network was considerably more robust to fixation in these primary cells, allowing much higher resolution of the mrtl-positive structures (Fig. 7).

As had been the case with the breast tumor cell lines, mrtl staining in the HMECs was most intense at the nuclear envelope. The continuity and tubular nature of the cytoplasmic ER network, where mrtl and DiOC6 colocalized, was much better appreciated in these cells, both in interphase (Fig. 7A) and mitotic cells (Fig. 7B). The three-way junctions and constant curvature of the membrane tubules seen in these cells are characteristic of the ER [Shibata et al., 2006].

The intranuclear membrane structures staining positively for mrtl (and DiOC₆) were also much better resolved. What we had consistently observed as a single mrtl-positive “target” spot positioned near the geometric center of the nucleus of the breast tumor cells was evident as a rather large and prominent cisternal reservoir within the nucleus of the HMECs. In addition, a network of small diameter intranuclear tubules which are both mrtl and DiOC₆ (i.e. membrane) positive was also seen. Together, this network of intranuclear membrane tubules and the central cisternal reservoir comprise the nucleoplasmic reticulum (NR), which has been described [Broers et al., 1999; Echevarria et al., 2003; Fricker et al., 1997; Johnson et al., 2003; Lagace and Ridgway, 2005] but is not widely appreciated as a significant intranuclear organelle. The nucleoplasmic reticulum is formed by deep invaginations of the nuclear envelope, and its contents are continuous with the cytoplasm.

We performed optical sectioning of the mrtl-positive structures in the primary HMECs to better establish the relationship between the tubular and cisternal elements of the nucleoplasmic reticulum (Fig. 7C–E). In the higher focal plane, a collection of small diameter membrane tubules can be seen extending from the nuclear envelope towards the interior of the nucleus (see arrow in yellow image). At the lower focal plane (~1.4 μ m nearer the substrate), the central cisternal reservoir is the dominant feature (arrow in red image). The tubular structures can be seen feeding directly into the central cisternal reservoir in the middle focal plane and overlay image. The NR provides an extensive increase in surface area for contact between the nucleus and cytoplasm. The prominent positioning of mrtl throughout the nuclear envelope and the NR suggests that mrtl could be involved in communication or transport between the cytoplasm and nucleus.

Ectopic overexpression of mrtl is accompanied by loss of Myc from the nucleus

Because mrtl and Myc are synthesized from the same mRNA molecule, we postulated that mrtl might function in cis to regulate translation and/or co-translational processing of Myc further downstream. The association of mrtl with components of the translational machinery, and its possible direct interaction with the *c-myc* mRNA, provided additional credence for this possible function of mrtl. To test this hypothesis, we examined Myc immunofluorescence in cells in which mrtl was ectopically overexpressed (Fig. 8).

Induction of ectopic mrtl expression was associated with a dramatic loss of Myc from the nucleus. Notably, this loss of nuclear Myc was observed throughout the field, and not restricted to cells with visibly elevated levels of ectopic mrtl, suggesting that the threshold for this effect of ectopic mrtl on Myc may be considerably lower than the threshold for visualization of ectopic mrtl by immunofluorescent imaging. Control transfectants (incapable of ectopic mrtl expression) treated with doxycycline did not exhibit this change in Myc staining pattern. The exclusion of Myc from the nucleus, where it serves as a transcription factor for RNA polymerases I, II, and III, would be expected to have a major impact on its function.

The most straightforward interpretation of this finding would be that mrtl negatively impacts the accumulation of Myc in the nucleus. However, we also noted that ectopic mrtl expression was accompanied by a dramatic decrease in intensity of staining for endogenous mrtl (Fig. 8G–J), suggesting that ectopic mrtl may function (unintentionally) as a dominant negative. This effect does not appear to be caused by competition between the two anti-mrtl antibodies, because a decrease in mAb131 reactivity accompanies induction of ectopic mrtl expression even when mAb102 is not used.

Ectopically expressed mrtl is also *wild-type* protein, appropriately localized to the nuclear envelope, ER, and NR membranes, and theoretically capable of equivalently replacing endogenous mrtl in the cell. However, because there is clearly an inherent limitation on the number of mrtl molecules which are appropriately post-translationally modified and

structurally integrated within the cell (as evidenced by the lower molecular mass of ectopic mrtl and its sensitivity to detergent extraction), the bulk of ectopically overexpressed mrtl is apparently not fully functional, and may participate in only a subset of the intermolecular interactions typical of endogenous mrtl. Furthermore, whereas endogenous mrtl is synthesized in cis to the *c-myc* mRNA, ectopic mrtl is generated in trans from the stably integrated tet-inducible construct, thus ectopic mrtl will not have the same opportunity to interact with the *c-myc* mRNA that endogenous mrtl has. Consequently, it appears that ectopically overexpressed mrtl may actually interfere with the normal regulatory function of endogenous mrtl, rather than amplifying it. By inference, endogenous mrtl may actually be a positive regulator of Myc, facilitating its translation and localization to the nucleus.

Disruption of C-terminal domain interactions displaces endogenous mrtl and decreases Myc protein levels

To confirm the relationship between mrtl and Myc, we elected to use a synthetic cell-permeable (myristoylated) peptide derived from the C-terminal interaction domain to (intentionally) dominantly interfere with the function of endogenous mrtl in untransfected cells (Fig. 9). This strategy to interfere with normal mrtl function is analogous to the use of RGD peptides to inhibit integrin function [Dresner-Pollak and Rosenblatt, 1994].

Cells treated with the myristoylated peptide exhibited multiple large, irregularly shaped cytoplasmic accumulations of mAb102-positive material, apparently representing the peptide itself. At the same time, the normal mAb131-positive distribution pattern of endogenous mrtl was almost completely obliterated. Total cellular mrtl levels were not altered with peptide treatment (western blot, Fig. 9G), thus it appears that endogenous mrtl may have been sequestered by the peptide into these cytoplasmic inclusions, and/or the peptide may be blocking epitope accessibility of mrtl from its native membrane locations.

Importantly, the normal nucleoplasmic staining of Myc was replaced by patchy, irregular cytoplasmic staining in the peptide treated cells, and western blot analysis revealed a marked, specific decrease in total Myc protein levels in these cells. Thus it appears that interference with normal mrtl function via sequestration and/or blocking of its intermolecular interactions has a negative impact on Myc. This result provides further evidence for a positive functional correlation between endogenous mrtl and Myc.

DISCUSSION

Detection of endogenous mrtl in human breast cells

We have presented definitive evidence for the natural existence of mrtl (previously hypothetical protein ORF1) in primary human breast epithelial cells and breast tumor cell lines. It is relatively rare in higher eukaryotes to find more than one distinct protein produced from a single genetic locus. One such precedent is the p16INK4a/p19ARF locus [Quelle et al., 1995; Sherr, 1998], in which overlapping protein coding sequences with distinct reading frames serve as substrates for synthesis of two proteins of completely unrelated primary amino acid sequence, yet related function (both tumor suppressors). Rather than utilizing alternate reading frames, the mrtl and Myc coding sequences are separated by ~800 nucleotides, with absolutely no overlap or homology, yet coexist within the same mRNA molecule. If our interpretation is correct, and mrtl is a positive modulator of Myc, then these two proteins may also share related (i.e. potentially oncogenic) function.

Intracellular localization of mrtl

Both ectopically expressed and endogenous mrtl were localized to the nuclear envelope, endoplasmic reticulum, and tubular and cisternal structures of the nucleoplasmic reticulum.

This pattern of membrane association of mrtl is consistent with the expectation that the hydrophobic N-terminal region would serve as a transmembrane domain. Localization to the nuclear envelope and nucleoplasmic reticulum places mrtl in an ideal position from which to influence cytoplasmic-nuclear communication and/or transport.

Molecular interactions of mrtl

The nuclear membrane and contiguous ER are studded with ribosomes, and in fact, mrtl colocalizes with translation regulatory molecules eIF2 α and eIF4G, as well as RACK1 (an integral component of the 40S ribosomal subunit [Gerbasí et al., 2004; Sengupta et al., 2004]), and co-immunoprecipitates with eIF2 α and RACK1. Furthermore, a dramatic enhancement of mrtl staining intensity and extractability is observed following nuclease digestion, suggesting that close association of mrtl with RNA may contribute to its tight structural integration in the cell. mrtl also appears to exhibit an RNA-binding activity of its own, interacting specifically with the 5'-untranslated sequences of *c-myc* (Fig. 6) as well as IGF1R (data not shown). Together, these findings suggest that mrtl may be involved in the translational regulation of *c-myc* and perhaps other mRNAs.

Functional relationship between mrtl and Myc

Ectopically overexpressed mrtl and the cell-permeable C-terminal peptide both appear to function as dominant negative inhibitors of endogenous mrtl function. Each is capable of only a subset of the intermolecular interactions of native mrtl: excess ectopic mrtl by virtue of the inherent limitations on post-translational modification and/or structural interaction partners, and the peptide by virtue of absence of the remainder of the mrtl protein. It is conceivable that failure of ectopic mrtl to interact appropriately with one or more components of the translational machinery could be responsible for its dominant negative effect on endogenous mrtl function, and the peptide may effectively block such interactions between endogenous mrtl and the translational machinery. In each case, we observed dramatic alterations to Myc, with loss from the nucleus, and even a decrease in total cellular Myc protein levels accompanying peptide treatment. Together these results seem to indicate a positive correlation between native mrtl and Myc, i.e. that the normal function of endogenous mrtl may be to facilitate Myc translation and localization to the nucleus.

Emerging data have begun to indicate the importance of regulation of *c-myc* expression at the translational level [Galmozzi et al., 2004; Hann et al., 1992; Notari et al., 2006]. The natural synthesis of mrtl in cis from the *c-myc* mRNA inherently places mrtl in the immediate vicinity of the regulatory sequences controlling the efficiency of Myc translation. A major determinant of *c-myc* translational regulation is the internal ribosomal entry site (IRES) [Le Quesne et al., 2001; Stoneley et al., 1998], positioned within the 5'-untranslated region between the mrtl and Myc coding sequences. One interesting possibility is that mrtl may regulate *c-myc* translation by modulating the activity of the IRES. Further experimentation will be required to determine whether this is the case.

It is well established that newly synthesized proteins which ultimately will function at the cell membrane are inserted into the ER membrane via interaction between the signal peptide and the signal recognition particle. It is conceivable that proteins such as Myc which are designated for function within the nucleus might be subject to an analogous co-translational trafficking event, whereby the nascent protein is directed through the nuclear membrane and into the nucleoplasm. The *c-myc* mRNA is known to be enriched in the perinuclear region [Mickleburgh et al., 2005], and the prominent positioning of mrtl at the nuclear membrane and nucleoplasmic reticulum (an extension of the nuclear-cytoplasmic interface), together with its overall homology (though weak) to the ATP-binding cassette (ABC) transporter proteins, are consistent with such a function.

Possible role of mrtl in Myc-associated oncogenesis

Myc is unequivocally implicated in the initiation and progression of a large proportion of human malignancies. From a genetic perspective, mrtl and Myc are very closely linked, with only ~800 bp separating their coding sequences, thus gene amplification or chromosomal translocations involving *c-myc* would frequently include the mrtl coding sequence as well. Considering the apparent functional relationship we have observed between mrtl and Myc, it is conceivable that mrtl may contribute significantly to the role of the *c-myc* locus in oncogenesis.

We had previously reported that ectopic expression of the 5'-untranslated region of the *c-myc* P0 transcript (including the intact mrtl coding sequence) in HeLa cells resulted in altered Myc protein levels and complete loss of tumorigenicity [Blume et al., 2003], along with a selective increase in the *c-myc* RNA-binding activity of a 15 kDa protein (unpublished data). In those earlier studies, as well as the present results, it appears that ectopically produced mrtl may have modified the regulatory status of *c-myc* in a complex (and incompletely understood) manner, with dramatic consequences for cellular phenotype.

Additional possible functions of mrtl

In addition to specific regulation of *c-myc* translation in cis, it is possible that mrtl may also function in trans to regulate the translational efficiency of other mRNAs. Clues that mrtl's physiological purpose in the cell may extend beyond modulation of *c-myc* include its widespread distribution throughout the ER/NR membrane network, its stable accumulation in cells, and its tight integration into the architecture of the cell. In fact, mrtl may play an important structural role in mediating the dynamic interactions between the intracellular membrane network, mRNA, and the translational machinery. Operating at the cytoplasmic-nuclear interface, mrtl may be very important for coordination of cytoplasmic processes with the highly orchestrated events occurring within the nucleus [Zaidi et al., 2007]. Further elucidation of mrtl function will be a high priority focus of ongoing investigations.

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Fig. 1. Architecture of the human *c-myc* locus and projected features of the *mrtl* protein
 (Above) The major landmarks associated with the human *c-myc* locus are indicated. The coordinates refer to base pairs, as established in the classical *c-myc* genomic sequence Accession X00364 [Gazin et al., 1984]. The positions of the four transcription start sites (P0, P1, P2, P3), each associated with a distinct DNase hypersensitive region of chromatin, and each apparently regulated independently, are shown as bent arrows. The three *c-Myc* exons are shown as rectangles, with the *c-Myc* coding sequences filled in. The CTG initiation codon for p67 (Myc1) is contained in exon 1, while the ATG initiation codon for p64 (Myc2) is contained in exon 2. The two ATG initiation codons for MycS are at positions 4821 and 4848 within exon 2. The coding sequence for MycHex1 is represented by the green dashed rectangle. The *mrtl* (ORF1) coding sequence, contained within the P0 transcript (accession M13930) but positioned well upstream of the *c-Myc* coding sequence, is represented by the red dashed rectangle. **(Below)** The major features anticipated for the hypothetical *mrtl* protein are shown. The coordinates refer to amino acids. The initiation codon for full-length *mrtl*, as well as an alternative translation initiation site potentially leading to production of a slightly smaller isoform of *mrtl* (designated *mrtx*), are indicated. The hydrophobic N-terminal region, expected to serve as a single transmembrane domain, is illustrated as a green hatched rectangle. The highly charged C-terminal region, a potential interaction domain, is illustrated as an orange wave-filled rectangle. The sequence of the 16-mer C-terminal peptide used to raise anti-*mrtl* antibodies is listed in blue text. Potential sites for serine phosphorylation and the kinases expected to act on them are shown in red. Possible sites for myristoylation and for tyrosine sulfation are also indicated.

Fig. 2. Analysis of ectopic and endogenous mrtl by western blot

Hot lysates (4% SDS, 99°C) or Cold lysates (1% NP-40, 4°C) were prepared from experimental (mrtl-expressing) or control clones with or without doxycycline (1 µg/ml × 3 days) induction. Equivalent amounts of protein were loaded onto 17.5% SDS/PAGE gels, transferred to nitrocellulose, and incubated with each of the anti-mrtl monoclonal antibodies (mAb131 **above**, mAb102 **below**). ORF17 and ORF33 are two independent clones ectopically expressing *wild-type* mrtl in a tet-inducible manner. dsko8 (double start knockout) is an otherwise tet-inducible clone in which both potential initiation codons of the ectopic mrtl sequence were mutated. Tet-On refers to the T47D cells expressing the reverse tet transactivator but not ectopic mrtl. Endogenous mrtl migrates with an effective molecular mass (~15 kDa) slightly larger than that of ectopic or in vitro translated mrtl (12.5 kDa), consistent with natural post-translational modifications likely to include myristoylation and serine phosphorylation. “mrtx”

is the designation for the N-terminal truncated isoform of mrtl which is generated by translation initiation at the internal methionine codon (also see Figure 1).

Fig. 3. Analysis of ectopic mrtl by immunofluorescence

(A–D) Experimental clones (ORF17, ORF33, or ORF43 (not shown)) capable of ectopically expressing mrtl were induced with doxycycline, fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 for 10 minutes, blocked in 10% goat serum, incubated with anti-mrtl mAb102 followed by AlexaFluor594-conjugated goat anti-mouse IgM (μ -chain specific), and visualized by laser scanning confocal microscopy. dsko8 (double start knockout) cells are incapable of ectopic mrtl protein expression. Bars, 50 μ m. (E–H) Higher magnification images of ectopically expressed mrtl in ORF17 cells. mrtl, red; actin, green; DAPI, blue. Bars, 20 μ m.

Fig. 4. Ectopic overexpression of mrtI is associated with accumulation of cells in G0

Protocol variations were used to explore the basis for heterogeneity of ectopic expression of mrtI. (A,D) Increase doxycycline to 2 $\mu\text{g/ml}$. (B,E) Increase Triton X-100 (permeabilization) to 0.5%. (C,F) Evaluate ectopic mrtI expression in colonies arising from single cells. (G–H) Treatment of cells with proteasome inhibitors ALLN or lactacystin. (I–J) Simultaneous staining of ectopic mrtI and Ki-67, with and without doxycycline induction. (K) Simultaneous staining of ectopic mrtI and BrdU (following incubation with 20 μM BrdU for 2 hours). mrtI, red; Ki-67 or BrdU, green; DAPI, blue. Bar, 50 μm .

Fig. 5. Endogenous mrtI colocalizes and co-immunoprecipitates with components of the translation machinery

Untransfected MCF-7 cells were simultaneously stained for mrtI (using mAb131, red) and (A–C) the lipophilic dye DiOC6, a marker of intracellular membranes; (D–F) Ribophorin II, a specific marker for the rough ER; and (G–I) eukaryotic translation initiation factor eIF4G. In J–X, the colocalization of endogenous mrtI with the integral 40S ribosomal protein RACK1 was examined in a series of human breast cell lines, including estrogen receptor-positive (T47D, MCF-7) and ER-negative (BT-20, MDA-MB-231) tumor cells, as well as one non-malignant breast epithelial cell line (MCF-10A). Bars in A–C and G–I, 20 μ m, bars in D–F and J–X, 50 μ m. (Y) co-IP of endogenous mrtI with eIF2 α and RACK1. Following preclearing with rabbit IgG or mouse IgM, whole cell lysates of untransfected T47D cells were incubated with monoclonal antibodies to eIF2 α or RACK1 bound to protein A/G-agarose or anti-IgM-agarose respectively, and the immunoprecipitates analyzed by western blot with mAb131 to mrtI. Micrococcal nuclease was included during the incubations to eliminate indirect co-IP through RNA.

A **B** **Fig. 6. Possible direct interaction of mrtl with the c-myc mRNA**

(A) T47D or MCF7 cells were subjected to hypotonic lysis in the presence of 1% NP-40 (yielding soluble cytoplasmic contents), followed by sequential treatment of the resuspended nuclear pellet with micrococcal nuclease and DNase I, 500 mM NaCl, and finally 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS. Equivalent proportions of each fraction were separated by SDS/PAGE and paired identical blots subjected to either northwestern analysis using the 5'-untranslated region of the c-myc mRNA (sense orientation) as probe, or western analysis using anti-mrtl mAb131. The results indicate that mrtl comigrates and co-fractionates with an RNA-binding protein that interacts specifically with the c-myc mRNA. (B) In situ biochemical fractionation of endogenous mrtl. MCF-7 cells grown on slides were subjected to hypotonic lysis with 0.25% NP-40 followed by digestion with micrococcal nuclease and DNase I prior to fixation and staining with mAb131 for mrtl as per standard protocol. The enhanced epitope accessibility following nuclease digestion is indicative of close association of mrtl with cellular nucleic acids (mRNA, possibly chromatin).

*Interphase**prometaphase*

Fig. 7. Endogenous mrt1 in normal primary human mammary epithelial cells: Distribution throughout the endoplasmic and nucleoplasmic reticular network

Normal primary human mammary epithelial cells (HMECs, derived from reduction mammaplasty) were cultured on slides, fixed and stained for endogenous mrt1 using mAb131. Confocal images of cells captured in (A) interphase or (B) prometaphase reveal an elaborate network of ER membrane tubules which are mrt1 (and DiOC₆, not shown) positive. (C–E) A series of optical sections of HMECs stained for endogenous mrt1 with mAb131 were captured at regular z-plane intervals, color-coded, and overlaid. The three images shown are each separated by 0.7 μ m, with E (red) nearer the substrate. The arrows point toward the interconnected tubular structures and central cisternal reservoir of the nucleoplasmic reticulum.

Fig. 8. Ectopic overexpression of mrtl is accompanied by loss of Myc from the nucleus (A,B,D,E) Double stable transfectants capable of ectopic expression of mrtl (ORF17 cells) were simultaneously stained for ectopic mrtl (red) and c-Myc (green), either with or without doxycycline induction. Cells shown in C and F were transfected with the parent vector and incapable of ectopic mrtl expression. Bars, 50 μm. (G–J) Ectopic (red) and endogenous (green) mrtl were simultaneously visualized in ORF17 cells, taking advantage of the differential epitope recognition properties of mAb102 and mAb131. Note the apparent inverse relationship between ectopic and endogenous mrtl revealed upon doxycycline induction.

Fig. 9. Dominant negative interference with endogenous mrtl function decreases Myc protein levels
(A–F) Untransfected cells were treated with a synthetic peptide derived from the C-terminal interaction domain of mrtl which had been modified by N-terminal myristoylation to enhance

cell permeability [Eichholtz et al., 1993]. After 48 hours, the effects on endogenous mrtI and Myc were assessed by immunofluorescence and confocal imaging. mAb102 detects the peptide (and possibly displaced endogenous mrtI). mAb131 detects endogenous mrtI in its native intracellular environment. **(G)** T47D cells treated with the myristoylated peptide (25 μ M daily) were harvested by hot lysis and analyzed by western blot for the status of mrtI (mAb131) and c-Myc (N262) protein. Lane “W” (washout), cells treated with peptide for 48 hours, then provided fresh media without peptide for an additional 48 hours.

Table 1

Properties of Ectopic and Endogenous mrtI

	Ectopic mrtI	Endogenous mrtI
Intracellular localization	Nuclear envelope; Endoplasmic/nucleoplasmic reticulum	Same as ectopic
Intercellular variability	heterogeneous	homogeneous
Recognition by mAb	102 > 131	131 > 102
Biochemical extraction	readily recovered- nonionic detergent	resistant, requires - nuclease, high salt
Epitope masking	not significant	substantial - uncovered by nuclease, mitosis
Post-translational modifications/Structural integration	likely limited by modifying enzyme (e.g. kinase) and/or interaction partner	serine phosphorylation; protein-protein interactions
Physical relationship to <i>c-myc</i> mRNA	in trans	in cis