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Growth Factors, Cytokines, Cell Cycle Molecules

Mortalin-Based Cytoplasmic Sequestration of p53 in a Nonmammalian Cancer Model

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In nature the soft shell clam *Mya arenaria* develops a fatal neoplasm that shares molecular similarity with an unrelated group of human cancers. In leukemic clam hemocytes, wild-type p53 and mortalin proteins co-localize in the cytoplasm. A similar phenotype, characterized by cytoplasmic sequestration of wild-type p53 protein, has been observed in several human cancers (undifferentiated neuroblastoma, retinoblastoma, colorectal and hepatocellular carcinomas, and glioblastoma). In some of these cancers p53 is tethered in the cytoplasm by mortalin when the latter protein is overexpressed. Using co-immunoprecipitation we have demonstrated that mortalin and p53 proteins are complexed in the cytoplasm of leukemic clam hemocytes (and not in normal hemocytes). In addition, treatment of leukemic clam hemocytes with MKT-077, a cationic inhibitor of mortalin, disrupts the interaction of mortalin and p53 proteins, resulting in translocation of some p53 to the nucleus. Based on these data, we introduce leukemic clam hemocytes as novel and easily accessible, *in vivo* and *in vitro* models for human cancers displaying a similar mortalin-based phenotype. Treatment of these models with novel chemotherapeutics may help reveal the molecular mechanism(s) involved in inactivating p53 by this form of cytoplasmic sequestration. ***(Am J Pathol 2006, 168:1526–1530; DOI: 10.2353/ajpath.2006.050603)***

Analyzing the effects of cytotoxic compounds on malignancy is currently limited to vertebrate models, which are expensive to maintain and highly regulated. Alternatively, studies of naturally occurring cancers in nonmammalian model organisms that are inexpensive to maintain can yield important data about molecular mechanisms that are held in common with human diseases.

A number of explanations have been proposed to account for the cytoplasmic sequestration of p53 in mammalian cancer cells. Among these are the overexpression of mortalin (a Hsp70 family member) and Parc (a parkin-like ubiquitin ligase) proteins that can independently serve as cytoplasmic tethers for p53.[1–3] The soft shell clam *Mya arenaria* displays a fatal leukemia at multiple sites along the coasts of New England and south to the Chesapeake Bay[4–6] (individuals from virtually any New England commercial source have 1% or greater incidence of this disease). The underlying molecular mechanisms that govern the development and progression of clam leukemia may reflect those seen in human diseases with similar mortalin-generated phenotypes.

Here we present the soft-shell clam as a naturally occurring, preclinical model for studies of mortalin-based cytoplasmic sequestration of wild-type p53 protein. In this study, we characterize normal (NCH) and leukemic (LCH) clam hemocytes *in vivo* and LCH *in vitro* using immunocytochemistry, co-immunoprecipitation, and subcellular localization of clam p53 (Map53) and clam mortalin (Mamot) proteins before and after treatment with the cationic inhibitor MKT-077.

Materials and Methods

Animals

Soft shell clams ($n \approx 70$ to 150) were collected at the lowest tides of each month from Marsh Island in New Bedford Harbor at Fairhaven, MA (41° 38.0′ N 70° 55.0′ W) and were maintained at the University of New Hampshire Coastal Marine Laboratory in New Castle, NH. To differentiate normal and leukemic clams, a small aliquot (10 μl) of hemolymph was aspirated from the pericardial sinus using a 26-gauge needle and incubated in 96-well microtiter plates for 2 hours at 8°C. Clams were classified using a Zeiss (Thornwood, NY) IM inverted microscope

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as normal (0% round, nonmotile LCH; 100% attached NCH), early incipient leukemic (1 to 50% LCH), late incipient leukemic (50 to 99% LCH), or fully leukemic (100% LCH). The average number of clams that was 100% leukemic in 51 collections was 5.64%; the range was 0 to 11% throughout a 5-year period.

LCH in Vitro

To prepare LCH for *in vitro* experiments, hemolymph was obtained from the pericardial sinus of freshly collected fully leukemic clams (100% leukemic) and then centrifuged. Pelleted LCH were resuspended in 10 ml of chemically defined culture medium[7] to which 10% heat-inactivated fetal bovine serum was added. LCH were seeded (4 to 7 $\times$ 10^4 ml^{-1}) in 100- to 150-ml spinner flasks at 32 rpm in an environmental chamber maintained at 8 to 10°C. Penicillin, ampicillin, and streptomycin were also added to all media at 200 U ml^{-1}, 25 μg ml^{-1}, and 200 μg L^{-1}, respectively.

Mamot Antibody Synthesis

A polyclonal antibody to Mamot was raised against a 21-residue peptide (NH2-CRLREAAEKAKIELSSSLQTD-COOH) synthesized by New England Peptide, Inc. (Gardner, MA) based on authentic Mamot sequence from a region containing residues within the MKT-077 binding domain (GenBank Accession number AY326398).[8] Bovine serum albumin was conjugated to the N-terminal cysteine using Imject maleimide-activated bovine serum albumin (Pierce Biotechnology Inc., Rockford, IL). The hapten-peptide conjugate was used to immunize two New Zealand White rabbits (Millbrook Farm, Amherst, MA). The antibody was affinity-purified with Affi-Gel (Bio-Rad, Hercules, CA) and screened for its ability to recognize Mamot protein using Western blot analysis.

Immunocytochemistry and Protein Localization in Clam Hemocytes

Cytospins of 100 μl of freshly collected hemocytes from normal (0% leukemic) and fully leukemic (100% leukemic) clams or of LCHs *in vitro* were fixed and permeabilized by immersion in acetone. Primary antibodies for immunocytochemistry (1:50 μl of primary antibody:buffer) included α-tubulin (clone DM-1A, mouse anti-chicken monoclonal from ICN, Irvin, CA; to demonstrate the microtubular array involved in cytoplasmic transport of Map53 protein),[9] clam mortalin (Mamot, rabbit anti-clam polyclonal), and clam p53 (Map53, rabbit anti-clam polyclonal).[10] Resulting preparations for α-tubulin were developed with the appropriate peroxidase-labeled secondary antibody (Vectastain ABC Elite IgG kit; Vector Laboratories, Burlingame, CA) followed by treatment with 3,3′-diaminobenzidine. To demonstrate co-localization of Mamot and Map53, polyclonal antibodies were directly conjugated to Quantum Dots 525 (green fluorescence, mortalin) and 655 (red fluorescence, p53) (Invitrogen, Carlsbad, CA). Control cytospins received identical treatment minus the conjugated antibody. LCHs were fixed in equal amounts of methanol and acetone, permeabilized in phosphate-buffered saline (PBS) containing 0.5% Triton X-100, blocked with PBS containing 0.05% Triton X-100 and 2% bovine serum albumin, and incubated for 12 hours in 0.5 μl of conjugated Mamot and 1 to 3 μl of conjugated Map53 antibodies (differences in amounts of antibodies relating to differences in preconjugation antibody concentrations). After rinsing the slides thoroughly with PBS containing 0.2% Triton X-100 and mounting the cells with polyvinyl alcohol-based mounting medium (Sigma-Aldrich, St. Louis, MO), fields of 200 cells were counted and scored as positive or negative on a Zeiss Axioplan II microscope equipped with epifluorescence, an AxioCam MR camera, and AxioVision 4.4 software (Carl Zeiss, Inc.).

To determine the distribution of Map53 protein between the nucleus and cytoplasm, 10 ml of normal and 1 ml of leukemic clam hemolymph (containing 1.5 $\times$ 10^8 LCH/ml^{-1}) were extracted from the pericardial sinus. Normal and leukemic hemolymph was centrifuged at 1200 rpm for 10 minutes. Hemocytes were isolated after centrifugation and nuclear and cytoplasmic proteins were extracted using an NE-PER kit (Pierce). Total protein was determined using a modified Lowry procedure. Distribution of nuclear and cytoplasmic Map53 protein in NCH and LCH cytoplasm was assayed by Western blot using our anti-Map53 polyclonal antibody; all lanes were loaded with 24 μg of total protein.[10]

Co-Immunoprecipitation of Mortalin and p53

Hemolymph was extracted from a leukemic clam (1 ml; 3.45 $\times$ 10^8 cells/ml) and a normal clam (5 ml; 1.55 $\times$ 10^6 cells/ml). Total cytoplasmic protein was extracted using the NEPER extraction kit (Pierce) and quantified using a modified Bradford procedure (Bio-Rad Laboratories, Hercules, CA). Co-immunoprecipitation of Map53 and Mamot was accomplished using an antibody-coupling gel to precipitate the bait protein (Map53) and co-immunoprecipitate the interacting prey protein (Mamot). Anti-p53 (Map53, rabbit anti-clam polyclonal) was coupled to an amine-reactive gel (ProFound co-immunoprecipitation kit, Pierce) using slow agitation at 22°C overnight. Cytoplasmic lysates for both NCH and LCH (100 μl) were incubated with the antibody-coupled gel in separate spin-columns. Two negative controls were also prepared, a control gel that used an inactivated form of the gel and a quenched gel, in which a quenching buffer was applied in place of the Map53 antibody. Both negative controls were incubated with a 50/50 mixture of NCH and LCH lysates and processed alongside the treatments in an otherwise identical manner. The first three elutions from each co-immunoprecipitation were stored for Western blot analysis. Elutions were separated by electrophore-

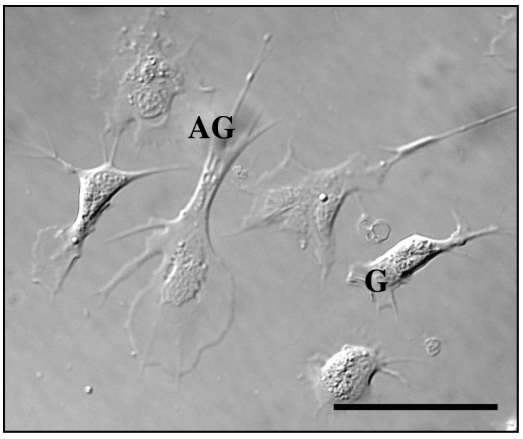

Figure 1. Nomarski image of living, freshly collected normal clam agranulocytic (AG) and granulocytic (G) NCH. Scale bar, 20 μm.

sis on a 4 to 15% Tris-HCl gel and then transferred to a polyvinylidene difluoride membrane. Concurrently, nuclear and cytoplasmic lysates from LCH were electrophoresed and analyzed as positive controls. The membrane was probed with Map53 antibody first to ensure presence of the bait protein followed by probing with Mamot antibody. Bands for Mamot were visualized using anti-rabbit horseradish peroxidase antibody and chemiluminescent detection reagents (Amersham, Arlington Heights, IL).

Treatment with MKT-077 and Disruption of Mortalin/p53 Co-Localization

To determine the appropriate concentration of MKT-077 to yield disruption of mortalin/p53 binding *in vitro*, MKT-077 at final concentrations of 1 to 7 μmol/L was added to a suspension of LCH. Cells were rotated at 1 rpm for 8 hours, prepared as cytospins, and assayed using direct observation of Romanovski-stained cells, the terminal dUTP nick-end labeling (TUNEL) assay (*In Situ* cell death detection kit, AP; Roche Laboratories, Indianapolis, IN) and simultaneous Quantum Dot immunocytochemistry for Mamot and Map53. Preparations were observed on a Zeiss Axioplan II microscope to determine the LC_{50} (concentration at which 50% of the cells died) and to detect translocation of Map53 into the nucleus.

Results

Distribution of p53 and Mortalin in NCH in Vivo

Normal clam hemolymph contained amitotic, terminally differentiated, agranular, and granular NCH (100% NCH at ~1 to 6 $\times$ 10^6 cells ml^{-1}) with an average diameter of 7 to 9 μm (Figure 1). Immunocytochemistry[10] and Western blotting (Figure 2) indicated positive reactions for Map53 protein in the cytoplasm and the nucleus of freshly collected NCH, whereas Mamot was only identified in the cytoplasm of NCH.

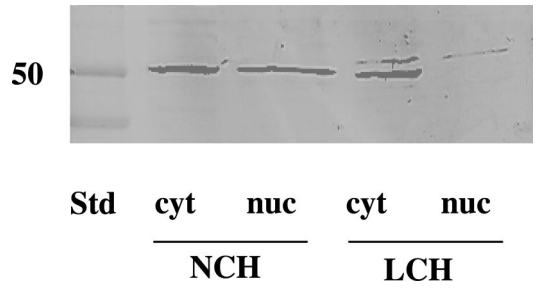

Figure 2. Western blot of NCH and LCH showing nuclear and cytoplasmic distribution of Map53 in the former and cytoplasmic sequestration of p53 in the latter. A larger p53 family member protein is also identified in LCH.

Co-Immunoprecipitation of Mortalin by p53

Presence of the bait Map53 protein was detected in cytoplasmic lysates from both NCH and LCH (data not shown). Co-immunoprecipitation using Map53 as bait demonstrated that complexes between Map53 and Mamot were found in cytoplasmic lysates from LCH but not in those from NCH (Figure 3). Both negative controls confirmed the validity of these results because neither produced a positive signal. Furthermore, detection using the Mamot antibody showed that the interaction occurred in LCH cytoplasm but not in the nucleus.

Distribution of p53 and Mortalin in LCH in Vivo *and* in Vitro

LCH *in vivo* (100% LCH at 5 $\times$ 10^8 cells ml^{-1}) had an average diameter of 7 to 10 μm (Figure 4a). These nearly round, mitotic hemocytes were monotonous in appearance and attached only loosely to plastic or glass. Results of immunocytochemistry of freshly collected LCH indicated positive reactions for α-tubulin (Figure 4b and inset), Mamot (Figure 4c), and Map53 (Figure 4d) proteins in the cytoplasm; none of these proteins was detected in the nucleus. Both Map53 and Mamot proteins co-localized near the centriole (perinuclear) or in relationship to the well developed microtubular array (pancytoplasmic); such overlap can be detected by the yellow color in Figure 4e. Western blot analysis of the distribution of Map53 confirms its sequestration in the cytoplasm in LCH (Figure 2). LCH *in vitro* were nearly round, averaged 5 to 7 μm in diameter, attached weakly to plastic and glass by short pseudopodia, and had obvious mitotic figures (Figure 4a). Distributions of α-tubulin, Mamot, and Map53 in the cytoplasm and nucleus of LCH *in vitro* were identical to those seen in LCH *in vivo*.

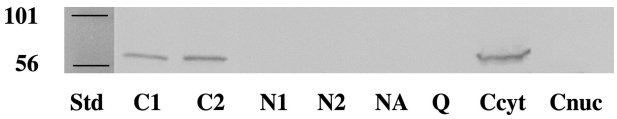

Figure 3. Co-immunoprecipitation of Mamot and Map53 in NCHs and LCHs. Std, protein standard; C1&2, first and second elute from LCH lysates; N1&2, first and second elute from NCH lysates; NA, first elute from inactive gel loaded with NCH and LCH lysate mix (negative control); Q, first elute from quenched gel loaded with NCH and LCH lysate mix (negative control); Ccyt, cytoplasmic protein extract from LCHs; Cnuc, nuclear protein extract from LCH.

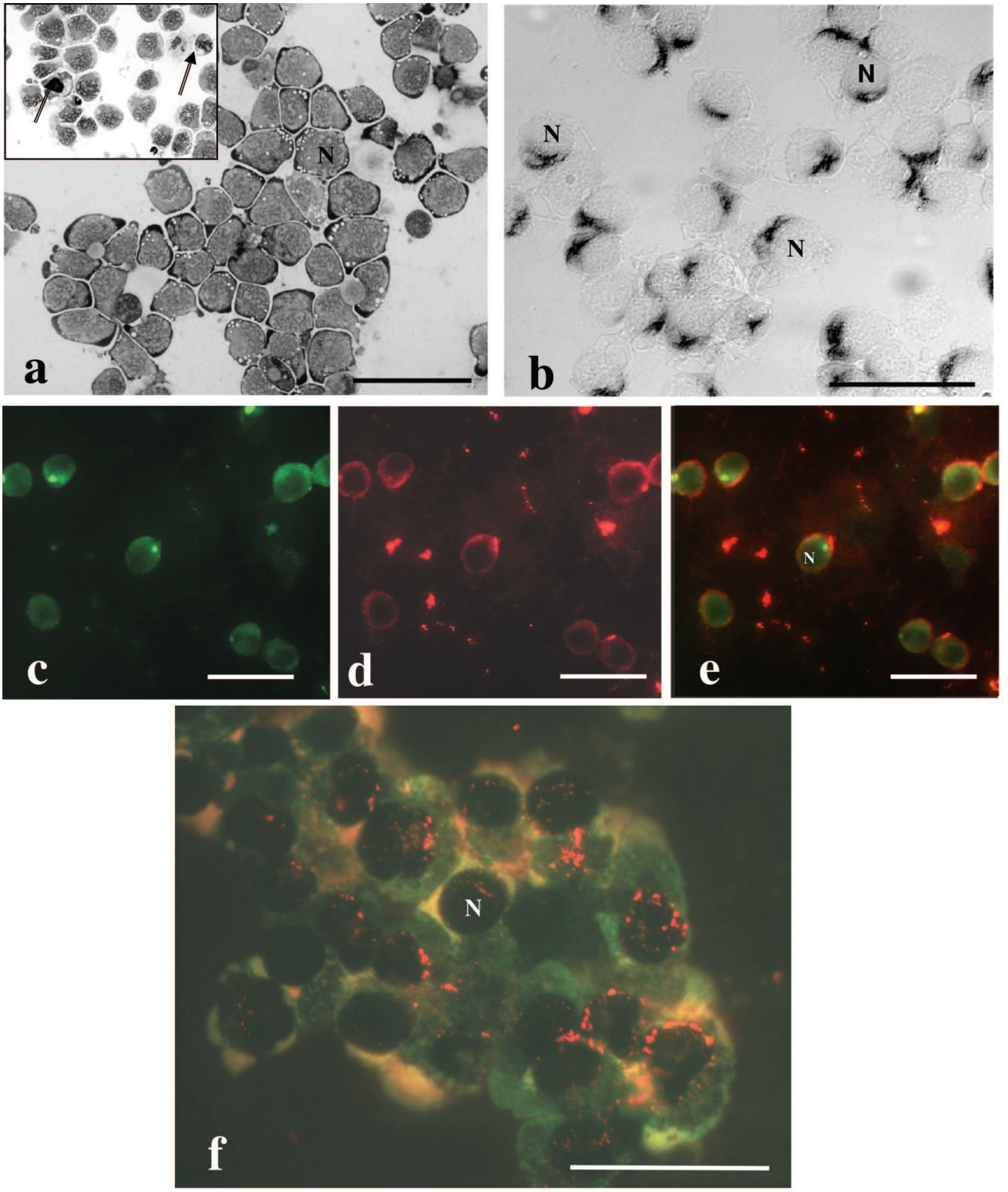

Figure 4. a: Romanovski-stained cytospin of freshly collected LCHs; **inset** shows similarly stained cytospin of LCHs maintained *in vitro* (**arrows** point out mitotic divisions). **b:** Cytospin of several LCHs showing localization of α-tubulin using indirect immunofluorescence (dark complex to one side of the nucleus). **c–e:** Quantum-dot immunocytochemical localization in LCH of Mamot (**c**, notice concentration near the centriole, **light green dot**), of Map53 (**d**) and merge to show cytoplasmic co-localization of Mamot and Map53 (**e**, yellow indicates co-localization of the two proteins). **f:** Disruption of Mamot and Map53 binding and translocation of some Map53 to the nuclei of cells treated with 3.5 μmol/L MKT-077. N, nucleus in all figures. Scale bars, 20 μm.

Treatment of LCH with MKT-077 Disrupts Mortalin/p53 Co-Localization

Treatment of LCH with 1 to 7 μmol/L MKT-077 displayed a dose-dependent increase in the translocation of Map53 protein into the nucleus at 6 to 8 hours (Figure 4f). A dose of 3.5 μmol/L MKT-077 was designated as the LC_{50} (50% of cells undergoing apoptosis based on observations from the TUNEL assay) (data not shown).

Discussion

In this study, immunocytochemistry, co-immunoprecipitation, and subcellular localization of clam p53 (Map53) and clam mortalin (Mamot) proteins before and after treatment with MKT-077 were determined for normal (NCH) and leukemic (LCH) clam hemocytes *in vivo* and *in vitro*. We show that Map53 and Mamot proteins formed complexes in the cytoplasm of LCH and not in NCH and that mortalin-based cytoplasmic sequestration of Map53 could be disrupted by MKT-077.

Cytoplasmic sequestration of Map53 protein is a characteristic feature of the phenotype of LCH,[10] and Map53 and Mamot are often concentrated near the centriole or mirror that of the well developed microtubular array. This distribution of Map53 protein reflects the pattern established by mortalin protein tethers in mice and humans. Cytoplasmic sequestration leads to inactivation of p53 family proteins in all of these organisms and is especially common in human lymphomas and neuroblastomas (up to 96% in undifferentiated neuroblastomas).[11,12] Cytoplasmic tethering of p53 is also mediated by mortalin and/or Parc protein anchors in undifferentiated neuroblastoma cells in mice and humans.[1–3]

In mammals, reversal of cytoplasmic sequestration of wild-type p53 protein can be achieved by a variety of means all of which result in translocation of p53 to the nucleus. Because these are wild-type proteins, their renewed presence in the nucleus reactivates them to direct p53-related transcriptional activities, usually resulting in apoptosis of the cancer cells. In all cases so far observed, reversal of cytoplasmic sequestration and resultant nuclear function of p53 protein requires either a reduction in the amount or availability of the cytoplasmic anchoring protein or in DNA damage-dependent overproduction and activation of p53 family proteins.

In leukemic clams, treatment with the rhodocyanin dye MKT-077 results in translocation of some Map53 protein from the cytoplasm to the nucleus and ultimately in apoptosis of LCH. Competition between Map53 and MKT-077 for the mortalin p53 binding site explains this response after treatment with MKT-077. This treatment results in a reduction of the effective concentration of Mamot in LCH. Because Map53 is sequestered in the cytoplasm, co-localizes with Mamot (which has a binding site for p53 that is 97% conserved with that of human mortalin), co-imunoprecipitates with Mamot, and is translocated to the nucleus after treatment with MKT-077, we suggest that Map53 is tethered in the cytoplasm of LCH by Mamot in a manner similar to that seen for p53 and mortalin proteins in a number of unrelated human cancers.

Acknowledgments

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