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Interaction of Microtubule-associated Protein 1B Light Chain (MAP1B-LC1) and p53 Represses Transcriptional Activity of p53

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Abstract: The tumor suppressor and transcription factor p53 is a key modulator of cellular stress responses, and can trigger apoptosis in many cell types including neurons. In this study, we have shown that Microtubule-associated protein 1B (MAP1B) light chain interacts with tumor suppressor p53. MAP1B is one of the major cytoskeletal proteins in the developing nervous system and essential in forming axons during elongation. We also demonstrate that both p53 and MAP1B-LC1 interact in the nucleus in HEK 293 cells. Indeed, we show that the MAP1B-LC1 negatively regulates p53-dependent transcriptional activity of a reporter containing the p21 promoter. Consequently, MAP1B light chain binds with p53 and their interaction leads to the inhibition of doxorubicin-induced apoptosis in HEK 293 cells. Furthermore, these examinations might be taken into consideration when knock-down of MAP1B-LC1 is used as a cancer therapeutic strategy to enhance p53's apoptotic activity in chemotherapy.

Key words: MAP1B-LC1, p53, transcriptional activity, apoptosis, protein interaction

Microtubule-associated protein 1B (MAP1B) is one of the major cytoskeletal proteins in the developing nervous system and essential in forming axons during elongation (Gonzalez-Billault et al., 2004; Riederer, 2007). It controls directions of growth cone migration, while neurons that lack MAP1B are characterized by increased terminal and collateral branching and impaired turning of growth cones (Bouquet et al., 2004; Garcia & Cleveland, 2001; Gonzalez-Billault et al., 2002; Takei et al., 2000). MAP1B consists of 2464 amino acids and synthesized as a polyprotein precursors which is rapidly cleaved to give rise to the respective heavy and light chains, termed MAP1B-HC and MAP1B-LC1 (Langkopf et al., 1992). The site of proteolytic cleavage of the MAP1B precursor has been narrowed down to a region in the proximity of amino acid 2210 (Togel et al., 1999). However, its cleavage proteolytic enzyme does not yet identified. Both MAP1B heavy and light chain has microtubule binding domain (MTB) and has the capacity to cross-link microtubules directly interacting with tubulin and actin (Riederer, 2007).

Because MAP1B interacts with varieties of other proteins, there is increasing evidence that MAP1B not only plays a crucial role in the stability of the cytoskeleton but also has other cellular functions. To maintain microtubule stability, MAP1B interacts with several other proteins such as gigaxonin, a protein that links microtubules and intermediate filaments (Allen et al., 2005; Ding et al., 2002), myelin associated glycoprotein (MAG), typical for cytoskeleton formation of glial cells (Dashiell et al., 2002; Franzen et al., 2001) and SCG10, essential in neurite outgrowth modulating microtubule disassembly (Bondallaz et al., 2006). Although most studies of MAP1B have focused on its interaction with microtubules, other roles have been proposed independent of microtubule-associated function. MAP1B directly binds with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), essential in the local energy provision of cytoskeletal structures, and help to keep this enzyme close to the cytoskeleton (Cueille et al., 2007). MAP1B also interacts with rho1 subunit of $GABA_C$ receptor at neuronal synapses (Billups et al., 2000; Hanley et al., 2000; Pattnaik et al., 2000). Anchoring to the cytoskeleton lowers the sensitivity of $GABA_C$ receptor in

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the inhibitory synapses and provides a functional modulation of the neuronal receptors. Recently, MAP1B light chain has been also identified as an interacting partner of Pes1 which is required for ribosome biogenesis and cell proliferation (Lerch-Gaggl et al., 2007). The interaction with MAP1B-LC1 induces a cytoplasmic sequestration of nucleolar Pes1, and that results in a reduction of cell proliferation. Thus, MAP1B-LC1 acts as a negative regulator of Pes1. Although MAP1B plays important roles in cytoskelectal stability and in other cellular functions, the exact underlying molecular mechanisms associated with MAP1B remains unclear.

The tumour suppressor protein p53 is involved in cell-cycle control, apoptosis, differentiation, DNA repair and recombination (Levine, 1997; Sengupta & Harris, 2005). In response to DNA damage, p53 accumulates in the nucleus (Harris & Levine, 2005), where it transcriptionally activates the genes that encode p21, BAX, PUMA and MDM2, as well as other genes that are involved in growth arrest and apoptosis (Kirsch & Kastan, 1998). In this study, we report that MAP1B-LC1 binds with p53 and inhibits transcriptional activity of p53 through their interaction. Therefore the present study was undertaken to investigate the possible effects of MAP1B-LC1 on doxorubicin induced apoptosis in the HEK 293 cells. Our findings suggest the MAP1B-LC1 acts as a negative regulator of p53 transactivation and doxorubicin induced apoptosis through their protein-protein interaction.

MATERIALS AND METHODS

Cell Culture and Transfection

HEK 293 cells were obtained from the ATCC (American type culture collection; Manassas, VA) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen; Carlsbad, CA) and penicillin-streptomycin (50 units/ml). Doxorubucin was purchased from Sigma Aldrich (St. Louis, MO). Transient transfection was performed by Lipofectamine 2000 (Invitrogen) with different plasmid DNA according to the manufacturer's instructions.

In Vivo Binding Assay and Western blotting

HEK 293 cells were seeded in 100 mm plates at an initial density of 2×10^6 cells and allowed to grow for 12 h. The cells were lysed in a buffer containing 1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.1% SDS, 1% Nonidet P-40, and 1 mM PMSF. The cell suspensions were incubated on ice for 20 min and centrifuged at 12,000 rpm at 4°C for 20 min. For immunoprecipitation assays, we used previously described protocols (Kim WH et al., 2007). The supernatants were pre-cleaned with 20 µl of protein A/G agarose bead (50% slurry) and then incubated at 4°C overnight with 30 ml of fresh protein A/G bead in the presence of appropriate antibodies. The beads were washed three times in PBS, resuspended in SDS sample buffer, and boiled for 10 min. Samples were analyzed by Western blotting using the appropriate antibodies to detect protein expression. The MAP1B and p53 protein were detected by each primary antibody (purchased from Santa-Cruz, 1:500 dilution) and secondary antibody (HRP-anti-goat and -anti rabbit, 1:5000 dilution). The polyclonal GFP, p21, Bax, PARP, α-tubulin and β-tubulin antibodies were from Santa Cruz Biotechnology. Western blot was detected by chemoluminescence (ECL, Santa Cruz Biotechnology).

Immunofluorescence Staining and Confocal Microscopic Detection

HEK 293 cells were grown on a sterile coverslips in 60 mm dishes and treated with doxorubicin. After the doxorubicin treatment, cells were washed with PBS and fixed for 1 h in 4% paraformaldehyde. The cells were permeabilized with 0.3% Triton X-100 for 20 min, incubated with a primary antibody in blocking solution for 3 h at room temperature, washed in blocking solution, and then incubated with the appropriate secondary antibody for 30 min. Primary antibodies were used at 1:200 for HA (sc-805: Santa Cruz). Cy3-conjugated goat anti-rabbit IgG (1:200, Amersham Biosciences) was used as secondary antibodies. Cells were visualized using a Radians 2000 confocal microscope (Bio-Rad).

Luciferase Assay

HEK 293 cells were cultured in 60 mm dishes and transfected with the firefly luciferase p21 reporter gene (0.1 µg) and pCMV-β-galactosidase (0.1 µg) together with pEGFP-MAP1B-LC, pcDNA-HA-53 and MAP1B siRNA (sc-35851: Santa Cruz) using Lipofectamine 2000. After 24 h of transfection, cells were lysed in reporter lysis buffer (Promega). Cell extracts were analyzed with the luciferase reporter assay system using a Lumat LB 9501 Berthold Luminometer. Luciferase activities of the p21-luciferase vector were normalized based on β-galactosidase activity of the cotransfected vector.

Preparation of Subcellular Fractions

HEK 293 cells were washed with ice-cold PBS, harvested by centrifugation, and lysed in Buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, and 1 mM phenylmethylsulfonyl fluoride) for 15 min. For cell lysis 10% (v/v) of Nonidet P-40 was added, and the cells were vortexed for 10 sec. After centrifugation at 5,000 rpm for 30 sec, the supernatant (cytosolic extracts) was transferred to a new tube. The pellet was added to ice-cold Buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM

DTT, and 1 mM phenylmethylsulfonyl fluoride), incubated for 15 min at 4 °C, and centrifuged at 14,000 rpm for 5 min. The supernatant (nuclear extracts) was transferred to new tubes and kept frozen at −70°C until use.

Apoptosis assays

To analyze cellular apoptosis, HEK 293 cells were grown to 60-70% confluence in complete media then treated with 2 μM doxorubicin in the presence or absence of a MAP1B-LC expression plasmid. Apoptotic cells were identified by their rounded morphology, compared to the spread-out morphology of non-apoptotic cells. Chromatin condensation as an apoptotic marker was visualized by Hoechst 33342 (Sigma-Aldrich, St. Louis, MO) staining.

RESULTS

p53 interacts with MAP1B-LC1 in HEK 293 cells

Using yeast two-hybrid assay, we have previously found that p73b, p53 homolog protein, binds with MAP1B light chain (unpublished our data). To examine whether MAP1B-LC1 can also interacts with p53, we co-transfected HEK293 cells with combined expression plasmids of GFP-fused-p53 with a HA-tagged MAP1B-LC1 expression vector, the whole cell lysates were immunoprecipitated with anti-HA antibodies. The immunoprecipitated proteins were then analyzed by Western blotting using specific anti-GFP and anti-HA antibodies to determine p53 and MAP1B-LC1, respectively (Fig. 1A). In the presence of MAP1B-LC1, the amount of co-immunoprecipitated p53 proteins was significantly increased (Fig. 1A, lane 2) compared to that in the absence of MAP1B-LC1 (Fig. 1A, lane 1). Conversely, HA-MAP1B-LC1 was also markedly immunoprecipitated with GFP-p53 (Fig. 1B, lane 2) but not with control GFP-empty vector (Fig. 1B, lane 1). For the control an almost even expression level of GFP-p53 and HA-MAP1B-LC1 in whole cell lysates was detected from each experimental condition, respectively (Fig. 1; total cell lysate). Taken together, these results demonstrate that p53 physically interacts with MAP1B-LC1 in cultured mammalian cells *in vivo*.

p53 binds with MAP1B-LC1 in the nucleus

To determine the localization of the HA-tagged p53 and GFP-fused MAP1B-LC1, we immuno-stained HEK 293 cells, which were co-transfected with HA-p53 and GFP-MAP1B-LC1 expression plasmids, using anti-HA antibodies. As shown in Fig. 2A, p53 (red) and MAP1B-LC1 (green) were co-localized in the nucleus under confocal microscopy. For further confirmation of the interaction between p53 and MAP1B-LC1 in the nucleus, we examined the nuclear co-localization and the interaction of the p53 and MAP1B-LC1 by Western blot analysis and immunoprecipitation. After HEK 293 cells were co-transfected with HA-tagged p53, GFP-fused-MAP1B-LC1 expression plasmids, both nuclear and cytoplasmic sub-fractions were separated and verified by Western blot analysis using antibodies for each marker protein, which were PARP for nucleus and β-tubulin for cytoplasm (Fig. 2B; bottom panel). The nuclear extracts were then immunoprecipitated with anti-HA antibodies,

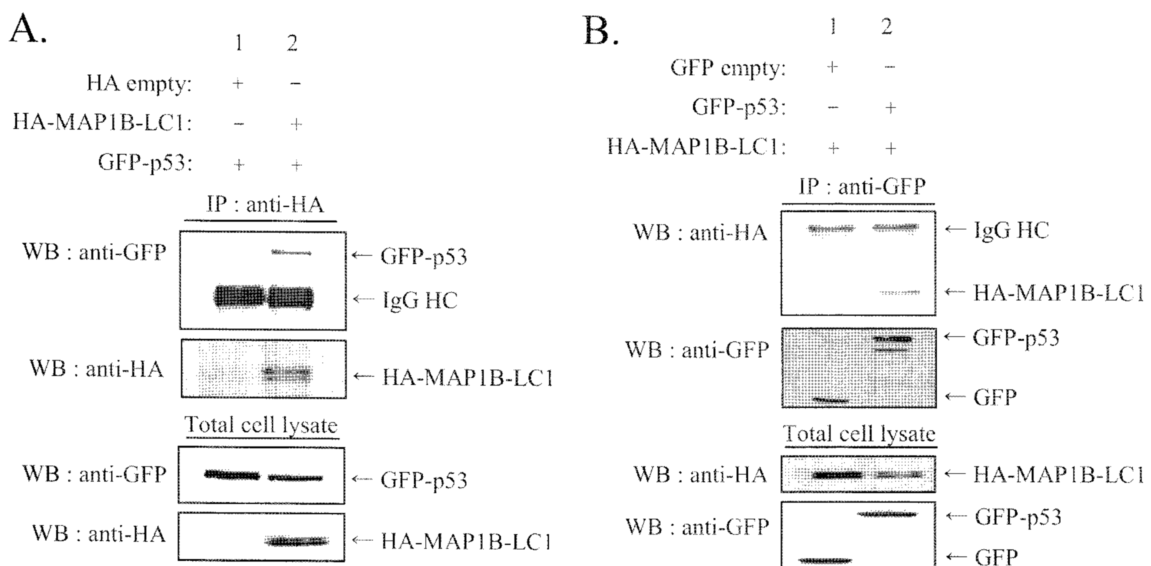

Fig. 1. MAP1B-LC interacts with p53 *in vivo*. (A) HEK 293 cells were transfected with expression plasmids of GFP-p53 together with either pcDNA-HA empty (lane 1), pcDNA-HA-MAP1BLC1 (lane 2). After whole cell lysates were immunoprecipitated with anti-HA antibody, western blot was performed using indicated antibodies. (B) HEK 293 cells were transfected with expression plasmids of pcDNA-HA-MAP1B-LC1 together with either pEGFP empty (lane 1), pEGFP-p53 (lane 2). After whole cell lysates were immunoprecipitated with anti-GFP antibody, western blot was performed using indicated antibodies.

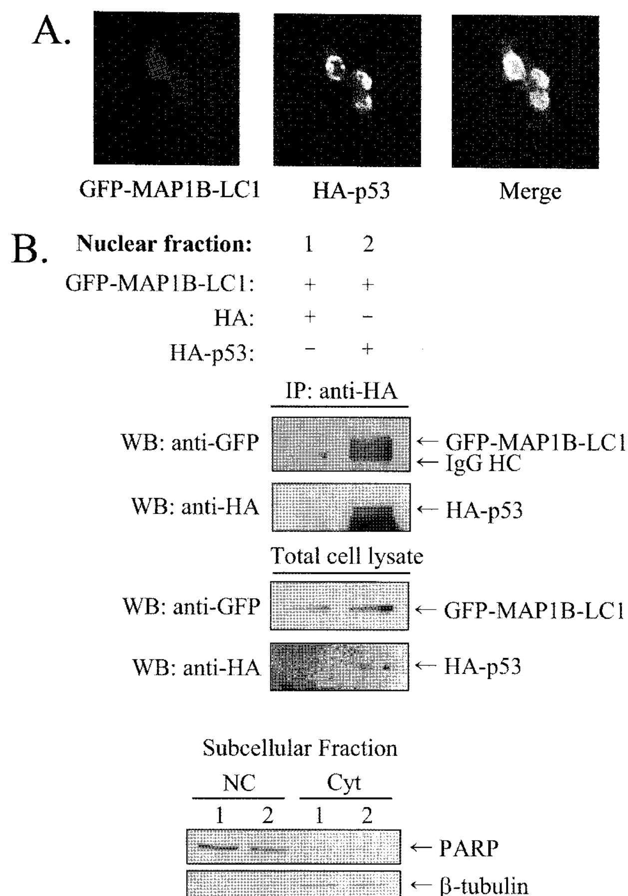

Fig. 2. p53 binds with MAP1B-LC1 in the nucleus. (A) HEK 293 cells were co-transfected with GFP-MAP1B-LC1 and pcDNA-HA-p53 expression plasmids. The cells were then immunostained and visualized green (GFP-MAP1B-LC1), red (HA-p53) under a confocal microscopic system as described in the "Materials and Methods". (B) HEK 293 cells were transfected with expression plasmids of GFP-MAP1B-LC1 together with either pcDNA-HA empty (lane 1), pcDNA-HA-p53 (lane 2). Their nuclear and cytoplasmic fractions were separately prepared as described in the "Materials and Methods". After nuclear fractions were immunoprecipitated with anti-HA antibodies, Western blot analysis was performed using anti-GFP and anti-HA antibodies. Their whole cell lysates served as controls. Either cytoplasmic or nuclear extracts were immunoblotted with anti-β-tubulin or anti-PARP antibodies, respectively, to ensure proper subcellular fractions.

and Western blot analysis was performed using anti-GFP and anti-HA antibodies. As indicated in Fig. 2B, GFP-fused MAP1B-LC1 was specifically immunoprecipitated with HA-tagged p53 in the nucleus fraction (Fig. 2B, lane 2) compared to HA-empty vector transfected group (Fig. 2B, lane1) even though all proteins were markedly detected in the total cell lysates. Therefore, we show that p53 specifically localizes and interacts with MAP1B-LC1 in the nucleus.

MAP1B-LC1 represses transcriptional activity of p53

To examine whether MAP1B-LC1 could affect p53-dependent transcriptional activation through their interaction, HEK 293 cells were transiently co-transfected with a p53 expression plasmid plus a luciferase reporter plasmid containing the p53-responsive element from the p21 promoter. As indicated in Fig. 3A, enhanced luciferase activities by p53 transactivation (lane 4) were repressed by MAP1B-LC1 expression in a dose dependent manner (lane 5, 6). Indeed, transfection of MAP1B siRNA into HEK 293 cells decreases repressive effect of both overexpressed GFP-MAP1B (Fig. 3B, lane 4) and endogenous MAP1B (Fig. 3B, lane 5) expression. Taken together, these results coherently suggest that MAP1B-LC1 could negatively regulate the transcriptional activity of p53.

The interaction between MAP1B-LC1 and p53 inhibits doxorubicin-induced apoptosis

Because MAP1B-LC1 inhibits transcriptional activity of p53, these results raise the possibility that MAP1B light chain may inhibit the doxorubicin-induced apoptosis. To investigate whether MAP1B-LC1 reduce doxorubicin-induced p53 activities, HEK 293 cells were transfected with MAP1B-LC1 and treated with 2 mM of doxorubicin. Then we examined the expression level of endogenous p21 and Bax, which is target genes of p53, by immunoblot analysis. As shown in Fig. 4A, the expression level of p21 and Bax was increased by doxorubicin treatment. However the increased p21 and Bax levels were reduced by MAP1B-LC1 overexpression (lane 3). These results suggest that MAP1B-LC1 represses doxorubicin-induced p53 activities by interaction of MAP1B-LC1 and endogenous p53. To determine whether MAP1B-LC1 inhibits doxorubicin-induced apoptosis, we examined the changes of cellular morphology by GFP empty vector transfection and nuclear condensation by Hoechst staining (Fig. 4B). After doxorubicin treatment for 24 h, the HEK 293 cells show characteristics of apoptotic morphology and chromatin condensation. However, doxorubicin treated cells expressing MAP1B light chain showed a normal morphology that diffusely stained intact nuclei (Fig. 4B, bottom panel). Taken together, these results indicate that MAP1B-LC1 interaction with p53 suppresses transcriptional activity of p53 and doxorubicin-induced apoptosis in HEK 293 cells *in vivo*.

DISCUSSION

In this study, we have shown that MAP1B light chain interacts with tumor suppressor p53, and it specifically binds with transactivation- and oligomerization-domain of p53 (unpublished our data). We also demonstrate that both p53 and MAP1B-LC1 co-localized in the nucleus in HEK 293 cells. Indeed, we demonstrate that the MAP1B-LC1 negatively regulates p53-dependent transcriptional activity of a reporter containing the p21 promoter. Consequently,

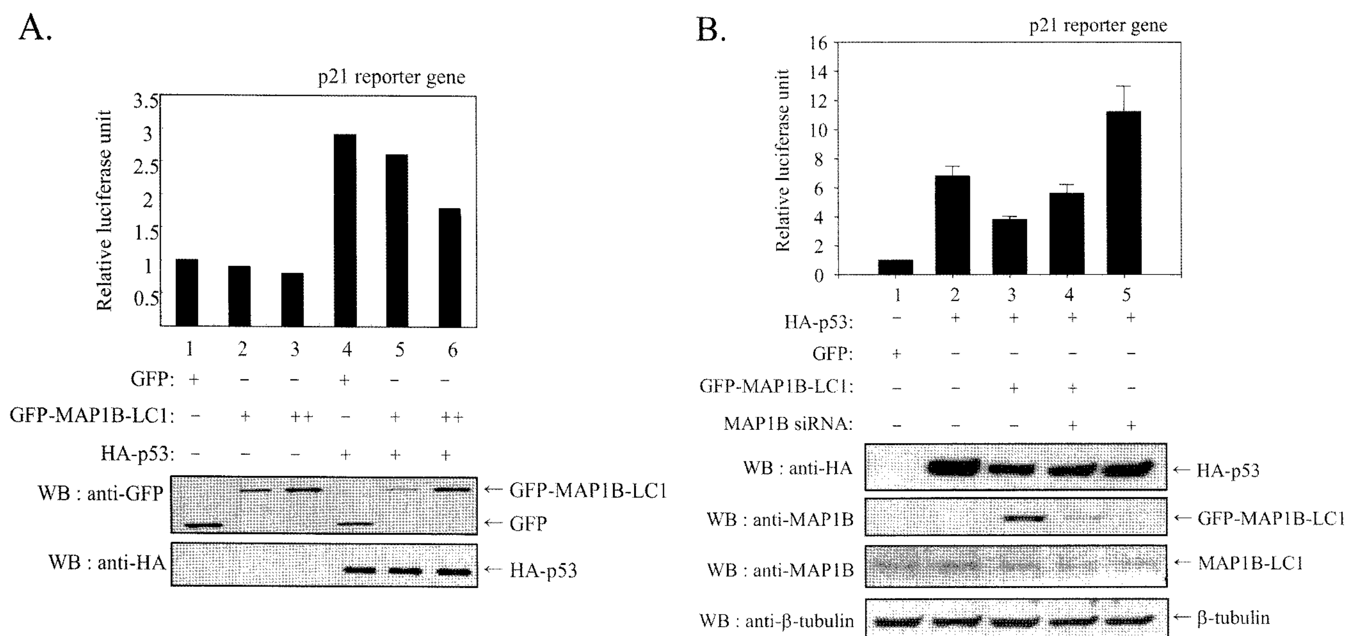

Fig. 3. MAP1B-LC represses transcriptional activity of p53. (A) HEK 293 cells were transiently co-transfected with GFP-empty, HA-p53 with increasing amounts of the GFP-MAP1B-LC1 expression plasmid (0.2, 0.4 μg). Luciferase activity was measured 36 h after transfection. All data were normalized to β-galactosidase activity. The data are expressed in relative fold increase of luciferase units (RLU). (B) HEK 293 cells were co-transfected with HA-tagged p53 and GFP-MAP1B-LC1 expression plasmids together with MAP1B siRNA. Luciferase activity was measured 36 h after transfection. All data were normalized to β-galactosidase activity. The data are expressed in relative fold increase of luciferase units (RLU). All data are representative of three independent experiments, and statistical analysis was represented by ±S.E.M (standard error meaning).

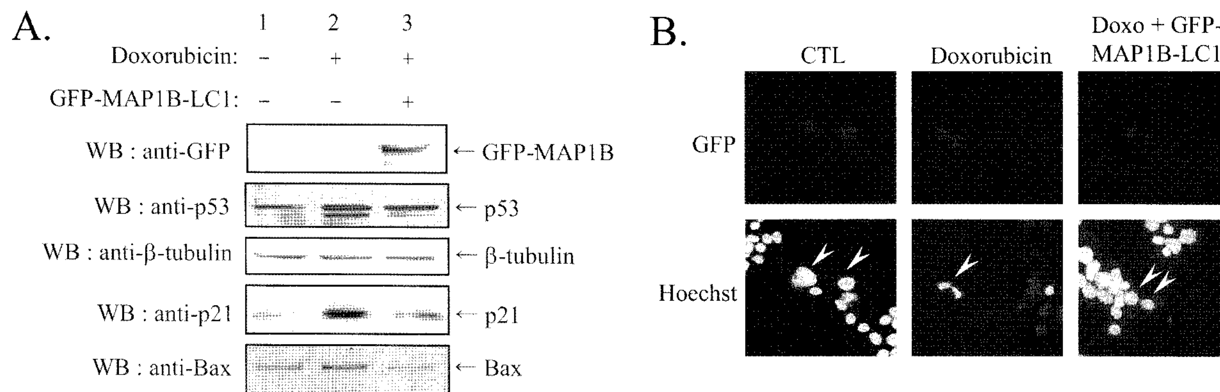

Fig. 4. MAP1B-LC1 inhibits doxorubicin-induced apoptosis in HEK 293 cells *in vivo*. (A) HEK 293 cells were transfected with GFP-tagged MAP1B light chain, and were treated with 2 μM doxorubicin for 24 h. Immunoblot analysis of protein levels of p53, p21 and Bax in cells were performed. β-tubulin levels are shown as loading control. (B) HEK 293 cells were transfected with GFP-MAP1B light chain, and treated with 2 μM doxorubicin for 24 h. And then cells were stained with Hoechst 33342 for detection of apoptotic cells.

MAP1B light chain binds with p53 and their interaction leads to the inhibition of doxorubicin-induced apoptosis in HEK 293 cells.

Previous studies have demonstrated that MAP1B not only form cross-links between individual microtubules for stabilizing microtubule structure but can interact with non-microtubule-associated proteins that also regulate microtubule stability (Riederer, 2007). It has been also reported that MAP1B has a novel another function (Lerch-Gaggl et al., 2007). As a novel function of MAP1B-LC1, it interacts with Pes1 and induces a cytoplasmic sequestration of nucleolar Pes1, and that results in a reduction of cell proliferation. Thus, MAP1B-LC1 acts as a negative regulator of Pes1.

Using yeast two-hybrid screening, we have identified a variety of p73β, p53 homolog, -interacting proteins, such as Amphiphysin IIb-1 (Kim et al., 2001) and p19^{ras} (Jeong et al., 2006) that are involved in p73β-mediated transactivity regulation and apoptosis. We also found the MAP1B-LC1 as a binding partner of p73β and confirmed their interaction *in vivo* and *in vitro* (unpublished our data). In addition, MAP1B-LC1 also can interact with p53 (Fig. 1) and

specifically binds with transactivation- and oligomerization domain of p53 (data not shown). Until now, there are several reports demonstrating that p53 has a potential of interaction with microtubule cytoskeleton (Giannakakou et al., 2000; Trostel et al., 2006). p53 associates preferentially with the polymerized form of tubulin (microtubules), and transported to the nucleus by the dynein motor complex. This interaction is lost after treatment with microtubule depolymerizing drugs. In turn, the association of p53 to microtubule allows for transport to the peri-nuclear region on microtubules, allowing for nuclear import.

Furthermore, we tested the physiological significance of binding between MAP1B light chain and p53 by assessing modulations in the transcriptional activity of p53. MAP1B-LC1 significantly decreased the activity of p53 (Fig. 3). Perhaps, MAP1B-LC1 might repress transcriptional activity of p53 through specific interactions with transactivation- and oligomerization domain of p53. MAP1B-LC1 binding to transactivation domain could prevent the association of co-activators, such as p300/CBP and PCAF to p53 and binding to oligomerization domain also inhibit the formation of activated p53-tetramer (Foo et al., 2007). Thus, our finding provide a molecular and functional linkage between microtubule-associated protein 1B (MAP1B) and p53 in cellular signaling.

Despite intensive functional studies, the effects of MAP1B on cellular physiology are most focused on microtubule related mechanisms and the other functions remain to be fully elucidated. In this aspect it is important to note that our findings contribute to a functional linkage between MAP1B and p53 in the biological network, showing that MAP1B-LC1 associates with and regulates p53 as a negative regulator in the HEK 293 cells. Furthermore, these examinations might be taken into consideration when knock-down of MAP1B-LC1 is used as a cancer therapeutic strategy to enhance p53's apoptotic activity in chemotherapy.

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