Degradation of Lung Adenoma Susceptibility 1, a Major Candidate Mouse Lung Tumor Modifier, Is Required for Cell Cycle Progression

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Abstract

We have previously identified murine lung adenoma susceptibility 1 (Las1) as the pulmonary adenoma susceptibility 1 candidate gene. Las1 has two natural alleles, Las1-A/J and Las1-B6. Las1 encodes an 85-kDa protein with uncharacterized biological function. In the present study, we report that Las1 is an unstable protein and the rapid destruction of Las1 depends on the ubiquitin-proteasome pathway. Las1 is a new microtubule-binding protein and Las1 associated with tubulin is not ubiquitinated. We further show that Las1-A/J is a more stable protein than Las1-B6. Las1 is expressed in the G2 phase of the cell cycle and that ubiquitin-proteasome–mediated Las1 destruction occurs in mitosis. Overexpression of Las1-A/J inhibits normal E10 cell proliferation and induces a defective cytokinesis. The differential degradation of Las1-A/J and Las1-B6 has important implications for its intracellular function and may eventually explain Las1-A/J in lung tumorigenesis.

Introduction

Lung cancer is the leading cause of death in the United States and worldwide (1). Lung cancer is largely induced by smoking; however, numerous studies also suggest there is a genetic basis for lung cancer (2, 3). Studies of lung tumorigenesis using inbred mouse system have revealed that the A/J mice is more susceptible than the C57BL/6J (B6) mice toward lung carcinogens, and this has permitted identification of quantitative trait loci (QTL) by linkage analysis (4, 5). The mouse pulmonary adenoma susceptibility 1 (Pas1) locus is one such QTL that has recently been fine mapped using congenic mice and shown to contain six candidate genes (4, 6). Systematic characterization of Pas1 candidate genes has limited the candidates to the lung adenoma susceptibility 1 (Las1) and Kras2 genes (4). Of these two, only Las1 displays an allelic variant that leads to an amino acid change at residue 60. Las1-A/J carries Asn60 and Las1-B6 carries Ser60. Preliminary studies have revealed that tumor cells expressing Las1-B6 exhibit slower rates of growth than Las1-A/J when cultured on plates and in xenograft mice (4). However, another study has shown that Las1-A/J inhibits cell growth compared with Las1-B6 (7). Therefore, the role of Las1 in lung tumorigenesis seems unclear and its molecular basis in lung cancer remains unexplored.

Ubiquitin-mediated degradation is the main nonlysosomal proteolytic pathway in eukaryotic cells (8). It plays a key role in eliminating misfolded proteins and disposing many short-lived regulatory proteins responsible for cell cycle progression, DNA repair, transcriptional regulation, signal transduction, apoptosis, and protein translocation (9, 10). Ubiquitin is an abundant and highly conserved 76-residue protein and is covalently attached to a target protein at lysine residues. Polyubiquitination of a protein marks it for degradation. Two discrete steps are involved in ubiquitin-mediated protein degradation: conjugation of multiple ubiquitin molecules to the target protein and degradation of the polyubiquitin-tagged substrate by the 26S proteasome (11). Conjugation of ubiquitin is carried out by a sequence of three enzymes: an E1 ubiquitin-activating enzyme, an E2 ubiquitin-conjugating enzyme, and an E3 ubiquitin ligase (12, 13). Ubiquitination of a specific substrate is mainly regulated through modulation of a degradation signal, such as phosphorylation of a target protein, or through control of the activity of its cognate E3, such as by association with a specific E3 activator (13).

Microtubules are important cellular cytoskeletal structure in eukaryotic cells and are involved in many cellular processes, including maintenance of cell shape, cell polarity, intracellular transport, and mitosis (14–16). Microtubules are highly dynamic structures equilibrating between α/β-tubulin dimers and α/β-tubulin polymers (microtubules). The polymerization dynamics is fundamentally important to the intracellular functions of the microtubule cytoskeleton. During the mammalian somatic cell cycle, microtubules undergo dramatic rearrangements from breakdown of cytoplasmic microtubules and subsequent formation of the mitotic spindle in the G2-M transition to dissolution of the spindle and reformation of the cytoplasmic microtubules on the return to interphase (17–19). In addition, microtubules interact with a large number of microtubule-associated proteins (MAP), which either regulate microtubule dynamics and physical properties or are motor molecules able to move in a unidirectional manner along the surface of microtubules (20). Microtubules also undergo various post-translational modifications and therefore distribute differently and associate with distinct sets of MAPs in cells (21).

Cytokinesis is the last stage of cell division for cytoplasm and cell cortex partition. The major event in cytokinesis is to build a cleavage furrow, an actin-myosin contractile ring, which constricts inwards to partition the parent cell into two daughter cells (22, 23). The actin cytoskeleton is a major player in cytokinesis because the cleavage furrow is formed by actin and myosin II; however, the correct positioning and assembly of the contractile ring requires the aster microtubules and mitotic spindle (22, 23). Therefore, completion of cytokinesis requires proper coordination from the microtubule cytoskeleton to the actin cytoskeleton and the cell membrane.

In this study, we describe the characterization of a novel mouse lung tumor susceptibility gene Las1. We show that Las1 is a new microtubule-binding protein and is a ubiquitinated protein but that tubulin-bound Las1 does not get ubiquitinated. Las1 undergoes cell cycle–dependent expression in the G2 phase. Las1-A/J and...
Las1 is not required for Las1 binding to h with anti-Las1 and anti-immunopellets were resolved by SDS-PAGE and analyzed by immunoblotting.

Hin pcDNA4-Las1-B6 in which sample buffer and sonicated. Samples from the supernatant (S) containing microtubules were dissolved in the same volume of SDS-PAGE. Las1, microtubule, and h Las1-B6. Cell extracts were incubated with anti-\(\beta\)-tubulin and anti-\(\alpha\)-tubulin antibodies, respectively. 293T cells were transiently transfected with vector, Las1-A/J, or Las1-B6. Preparations of lysates, anti-\(\beta\)-tubulin immunoprecipitation, and anti-Las1 immunoprecipitation were done as above. C, Las1 cosediments with microtubules. 293T cells were transiently transfected with vector, Las1-A/J, or Las1-B6. Cells were lysed with a microtubule-stabilizing buffer. After centrifugation, pellets containing microtubules were dissolved in the same volume of SDS-PAGE sample buffer and sonicated. Samples from the supernatant (S) and pellet (P) fractions were boiled and resolved by SDS-PAGE. Las1, \(\alpha\)-tubulin, and \(\beta\)-tubulin were visualized by immunoblotting analyses with anti-Las1, anti-\(\alpha\)-tubulin, and anti-\(\beta\)-tubulin antibodies, respectively. D, Las1 colocalizes with microtubules in interphase cells. NIH3T3 and E10-3 cells were grown on coverslips. NIH3T3 cells on the right column were treated in culture with 2 \(\mu\)g/mL nocodazole (Noc) for 1 h. All cells were then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then stained for \(\alpha\)-tubulin (green), \(\beta\)-tubulin (red), and nucleus (blue), as indicated.

Las1-B6 display differential degradation by the 26S proteasome in mitosis with Las1-A/J being slower than Las1-B6. Consequently, Las1-A/J induces a cytokinesis defect in a normal immortal lung epithelial cell line. These observations lead us to hypothesize that Las1-A/J disturbs microtubule function in the cytokinesis phase of the cell cycle. Our results provide further insight into the role of Las1 in lung tumorigenesis.

Materials and Methods

Plasmid constructs and mutagenesis. Vectors pcDNA4-Las1-A/J and pcDNA4-Las1-B6 in which Las1-A/J or Las1-B6 allele was cloned into the HindIII and EcoRI sites of the pcDNA4/TO vector (Invitrogen). Vector pcDNA3-Flag-ubiquitin encoding an NH\(_2\)-terminal Flag-tagged human ubiquitin was provided by Dr. K-L. Guan (University of Michigan, Ann Arbor, MI).

Cell culture and transfection. COS-7, 293T, and NIH3T3 cells were grown in DMEM (Mediatech, Inc.) plus 10% (v/v) fetal bovine serum (FBS; Sigma). E10, E10-2, and E10-3 cells were cultured in CMRL 1066 (Invitrogen) plus 10% FBS and 2 mmol/L glutamine (Mediatech). Cells were seeded in six-well plates with 60% confluence, cultured overnight, and transfected with indicated plasmids unless specified otherwise. All transfections were done using LipofectAMINE 2000 reagent (Invitrogen). To generate stable Las1 cell lines, pcDNA4/TO (vector), pcDNA4-Las1-A/J (Las1-A/J), and pcDNA4-Las1-B6 (Las1-B6) were linearized with PvuI and transfected into E10 cells. Cells were selected with 500 \(\mu\)g/mL zeocin (Invitrogen), and the resistant colonies were maintained in 100 \(\mu\)g/mL zeocin.

Immunoprecipitation and immunoblotting. Cells were lysed using lysis buffer [100 mmol/L Tris-HCl (pH 7.4), 200 mmol/L NaCl, 10 mmol/L EDTA, 10% sucrose, 1.25% NP40, protease and phosphatase inhibitors]. Lysates were precleared with protein A-Sepharose (Amersham) for 1 h at 4°C and incubated with indicated primary antibodies. Immunoprecipitates were washed and resolved by SDS-PAGE, electrophoresed onto 0.45 \(\mu\)m filters.

Figure 1. Interaction of Las1 and tubulin. A, Las1 coimmunoprecipitates with \(\beta\)-tubulin. 293T cells were transiently transfected with vector, Las1-A/J, or Las1-B6. Cell extracts were incubated with anti-\(\beta\)-tubulin, antibody, and immunopellets were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Las1 and anti-\(\beta\)-tubulin antibodies, respectively. B, residue 60 on Las1 is not required for Las1 binding to \(\beta\)-tubulin. 293T cells were transiently transfected with vector, Las1-A/J, Las1-B6, or pcDNA4-Las1-\(\Delta60-69\). Preparation of lysates, anti-\(\beta\)-tubulin immunoprecipitation, and anti-Las1 immunoblotting was done as above. C, Las1 cosediments with microtubules. 293T cells were transiently transfected with vector, Las1-A/J, or Las1-B6. Cells were lysed with a microtubule-stabilizing buffer. After centrifugation, pellets containing microtubules were dissolved in the same volume of SDS-PAGE sample buffer and sonicated. Samples from the supernatant (S) and pellet (P) fractions were boiled and resolved by SDS-PAGE. Las1, microtubule, and \(\beta\)-tubulin were visualized by immunoblotting analyses with anti-Las1, anti-\(\beta\)-tubulin, and anti-\(\alpha\)-tubulin antibodies, respectively. D, Las1 colocalizes with microtubules in interphase cells. NIH3T3 and E10-3 cells were grown on coverslips. NIH3T3 cells on the right column were treated in culture with 2 \(\mu\)g/mL nocodazole (Noc) for 1 h. All cells were then fixed with 4% paraformaldehyde, permeabilized with 0.5% Triton X-100, and then stained for Las1 (red), \(\beta\)-tubulin (green), and nucleus (blue), as indicated.

Figure 2. Mapping the tubulin interaction domain of Las1. A, schematic representation of Las1 and Las1 truncation mutants and a summary of the interaction between Las1, Las1 truncation mutants, and \(\beta\)-tubulin. Shaded box, a predicted coiled-coil region; numbers on the left, residues of Las1 retained in each mutant. , an internal deletion; +, positive interaction determined by reciprocal communoprecipitation (Co-IP); -, negative interaction determined by reciprocal communoprecipitation. ND, not determined. B, expression of Las1 and Las1 mutant proteins. 293T cells were transiently transfected with vector, Las1-A/J, and Las1-A/J truncation mutants listed in A. The same amounts of cell lysates from each transfection were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Las1 and anti-\(\beta\)-tubulin (internal loading control) antibodies, respectively. C, reciprocal communoprecipitations of Las1-A/J or Las1-A/J mutants and \(\beta\)-tubulin. The cell lysates described in B were immunoprecipitated (IP) with anti-\(\beta\)-tubulin followed by anti-Las1 immunoblotting (IP; top) or anti-Las1 antibody immunoprecipitation followed by anti-\(\beta\)-tubulin communoprecipitation analyses (bottom). *, nonspecific bands.
polyvinylidene difluoride membrane, and probed with indicated primary followed by secondary antibodies. Final visualization of protein signals was done with the enhanced chemiluminescent kit (Pierce).

**Microtubule cosedimentation assay.** Microtubule cosedimentation assay was done according to the protocol of Vos et al. (24). Briefly, cells were washed twice with PBS, lysed with a hypotonic buffer [20 mmol/L Tris-HCl (pH 6.8), 1 mmol/L MgCl₂, 2 mmol/L EGTA, 0.5% NP40, protease and phosphatase inhibitors] for 5 min, and centrifuged at 15,000 rpm for 10 min. Supernatant and pellet were resolved by SDS-PAGE, and associations of Las1-A/J and Las1-B6 were determined by immunoblotting using anti-Las1 antibody. Ubiquitin-Las1 conjugates were detected by immunoblotting with anti-ubiquitin antibody.

**Immunoﬂuorescence and microscopy.** Exponentially growing cells were seeded on 22-mm coverslips in six-well plates at 50% confluence and cultured for 24 h. To depolymerize microtubules, nocodazole was added to the culture with a final concentration of 5 μg/mL for 1 h. Double immunofluorescence staining was done as follows. Cells were fixed with 4% paraformaldehyde/PBS for 10 min and permeabilized with 1% Triton X-100/PBS for 10 min. The cells were then blocked with 3% bovine serum albumin/paraformaldehyde/PBS for 10 min and permeabilized with 1% Triton X-100/PBS for 1 h. Coverslips were stained with 4',6-diamidino-2-phenylindole (DAPI) and mounted on slides. Images were captured by Olympus DP70 digital camera and analyzed using Olympus MicroSuite FIVE imaging software.

**Measurement of Las1 half-life.** COS-7 cells were transiently transfected with 0.5 μg Las1-A/J or Las1-B6. At 36 h after transfection, cells were treated with 100 μg/mL cycloheximide (Sigma). Cells were collected at the indicated time point and lysed with the lysis buffer. Lysates (20 μg) were resolved by SDS-PAGE and analyzed by immunoblotting with anti-Las1 and anti-β-tubulin antibodies. Films were scanned and the intensities of bands were analyzed using the software ImageJ, version 1.36b.

**In vivo ubiquitination assay.** COS-7 cells were transiently transfected with 1 μg pcDNA3-Flag-ubiquitin along with 1 μg of vector, Las1-A/J, or Las1-B6, respectively. At 36 h after transfection, the cells were treated with 100 μg/mL cycloheximide and harvested at the indicated time points. Cell lysates (20 μg) from each sample were analyzed with immunoblotting using anti-Las1 and anti-β-tubulin antibodies, respectively. The intensity of each band was digitalized by ImageJ software and normalized against β-tubulin at the same time point. The graph represents the remaining percentage of Las1 at the indicated time point by setting the remaining percentage at zero time point as 100%.

Results are from one representative experiment of two done.

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**Figure 3.** Ubiquitin-proteasome mediates Las1 destruction. A, Las1 is ubiquitinated (Ub) and that tubulin-associated Las1 is not ubiquitinated. 293T cells were transiently transfected with Las1-A/J (left) or Las1-A/J along with pcDNA3-FLAG-ubiquitin (right). Cell lysates were immunoprecipitated with anti-Las1 followed by immunoblotting with anti-ubiquitin (left) or anti-FLAG antibodies (right), respectively. B, ubiquitinated Las1 is destructed through the 26S proteasome. COS-7 cells were transiently transfected with 1 μg of vector, Las1-A/J, or Las1-B6. At 36 h after transfection, 20 μmol/L of MG132 or DMSO were added to the culture for additional 12 h. Cell lysates were subjected to immunoprecipitation using anti-Las1 antibody. Immunoprecipitates were analyzed by immunoblotting with anti-ubiquitin antibody. C, Las1-A/J is more stable than Las1-B6. COS-7 cells were transiently transfected with 1 μg of vector, Las1-A/J, or Las1-B6 along with or without 1 μg pcDNA3-FLAG-ubiquitin. At 36 h after transfection, cells were lysed and the same amounts of cell lysates were subjected to immunoblotting for anti-Las1 and anti-β-tubulin antibodies, respectively. D, half-life of Las1-A/J and Las1-B6. COS-7 cells were transiently transfected with 0.5 μg of Las1-A/J or Las1-B6. At 36 h after transfection, the cells were treated with 100 μg/mL cycloheximide and harvested at the indicated time points. Cell lysates (20 μg) from each sample were analyzed with immunoblotting using anti-Las1 and anti-β-tubulin antibodies, respectively. The intensity of each band was digitalized by ImageJ software and normalized against β-tubulin at the same time point. The graph represents the remaining percentage of Las1 at the indicated time point by setting the remaining percentage at zero time point as 100%.

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1 Bioprotocols (http://www.bio.com/protocoltools/).
Results

Interaction of Las1 and tubulin. We sought to explore Las1 functions by determining which proteins form complexes with Las1. We identified tubulin as a binding protein of Las1-A/J and Las1-B6 using standard pull-down assay by passing through glutathione 5-transferase (GST), GST-Las1-A/J, and GST-Las1-B6 glutathione-agarose beads with A/J mice lung extracts (Supplementary Fig. S1). We chose coimmunoprecipitation assay to confirm the interaction of Las1 and tubulin. As shown in Fig. 1A, both Las1-A/J and Las1-B6 were detected in the anti-β-tubulin immunoprecipitates but not in the absence of transfected Las1, confirming that the interaction of Las1 and tubulin is specific. There was less Las1-B6 than Las1-A/J coimmunoprecipitated with β-tubulin. We did reciprocal coimmunoprecipitation assays and got similar results (Supplementary Fig. S2).

We found that the same amounts of transfected expression constructs produced larger amounts of Las1-A/J than Las1-B6 despite the fact that these constructs differ only by one residue in the Las1. To investigate if there are some differences in Las1-A/J and Las1-B6 in binding to tubulin, we generated a mutant Las1Δ11-69 in which residues 11-69, covering the predicted coiled-coil region and the single nonsynonymous residue between Las1-A/J and Las1-B6, were deleted. As shown in Fig. 1B, besides Las1-A/J and Las1-B6, Las1Δ11-69 also coimmunoprecipitated with β-tubulin, suggesting that the coiled-coil region on Las1 is not required for tubulin binding. Therefore, Las1-A/J and Las1-B6 bind to tubulin equally.

Cosedimentation of Las1 and microtubules. To further distinguish whether Las1 is associated with tubulin and/or microtubules, we determined whether Las1 can coprecipitate with microtubules. 293T cells were transiently transfected with empty vector and vectors encoding Las1-A/J and Las1-B6. Cells were lysed in a low-salt lysis buffer to stabilize microtubules. In this assay, proteins associated with microtubules are captured in the pellet fraction with microtubules. As shown in Fig. 1C, Las1-A/J and Las1-B6 were in the pellet fractions with microtubules. These results show that both Las1-A/J and Las1-B6 associate with microtubules.

Coimmunolocalization of Las1 and microtubules in interphase cells. A previous study using Myc-tagged Las1 localized Las1 to the cytosol in NIH3T3 and COS-7 cells with a diffuse staining pattern (4). In the current study, we immunostained Las1 and β-tubulin in NIH3T3 and E10-3 cells, a stable Las1-B6 cell line in which Las1-B6 is overexpressed in E10 cells. As shown in Fig. 1D, anti-Las1 immunostaining revealed a distribution of Las1 that colocalized with the microtubule cytoskeleton. To analyze whether the localization of Las1 is dependent on the integrity of microtubule cytoskeleton, we treated cells with nocodazole, a microtubule-depolymerizing drug. Our results revealed that disruption of the microtubule cytoskeleton completely abolished the Las1 fiber-like staining pattern. This result indicates that intact microtubules are required for Las1 association and further confirms that Las1 is associated with the microtubule network.

Identification of an internal Las1 fragment bound to β-tubulin. The Las1 cDNA encodes a 730-residue protein. There is no known functional domain besides a predicted coiled-coil motif present at the NH₂ terminus. As both α/β-tubulin [isoelectric point (pI) 4.8/5.3] and Las1 (pI 5.3) are acidic proteins, any nonspecific binding via electrostatic interactions is unlikely. We chose Las1-A/J to delineate the tubulin-binding region of Las1. We generated a series of truncation mutants of Las1-A/J (Fig. 2A), and all these mutants but Las1 (195-263) were expressed (Fig. 2B). Interactions of the expressed Las1 mutants and tubulin were determined by coimmunoprecipitation assays. As shown in Fig. 2C, among the seven Las1 constructs examined, Las1 (1-263) was the shortest NH₂-terminal fragment tested positive in the interactions. Las1 (1-194) did not interact with β-tubulin, whereas Las1 (195-730) did interact with β-tubulin. These results suggest that the interaction domain might locate within residues 195-263 of Las1. Interestingly, Las1 (195-263) has a pI 9.3 and is very likely to be a region to interact with β-tubulin (pI 5.3). Unfortunately, there was no detectable expression of Las1 (195-263). Finally, Las1 (195-381) interacted with β-tubulin (Supplementary Fig. S3), which suggests that the residues 264-381 of Las1 may not be necessary for β-tubulin binding but may influence the stability or folding of Las1, particularly in the context of the smaller Las1 (195-263) fragment. Taken together, we conclude that the Las1 (195-381) is necessary and sufficient for β-tubulin binding.

Tubulin-associated Las1 is not ubiquitinated. The higher molecular weight smears of Las1 shown in Fig. 2B suggest that Las1 might be polyubiquitinated. To confirm this, anti-Las1 immunoprecipitates (Fig. 2C, bottom) were probed with anti-ubiquitin antibody and displayed robust polyubiquitin signals (Fig. 3A, left), indicating that Las1-A/J is a ubiquitinated protein. However, there were no detectable ubiquitin-Las1 signals from the Las1 coimmunoprecipitated with β-tubulin (Fig. 2C, top). These observations seem to suggest that Las1 associated with β-tubulin is not ubiquitinated. To confirm this, 293T cells were transiently cotransfected with pcDNA3-Flag-ubiquitin along with Las1-A/J or Las1-B6. Anti-Las1 immunoprecipitation followed by anti-Flag immunoblotting revealed robust Flag-ubiquitin-Las1 signals (Fig. 3A, right), whereas anti-β-tubulin immunoprecipitation followed by anti-Flag immunoblotting did not reveal any similar high molecular weight smears even after a much longer exposure (Supplementary Fig. S4, left). Las1 coimmunoprecipitated with β-tubulin was confirmed by anti-Las1 immunoblotting (Supplementary Fig. S4, right). These results confirm that Las1 is a
ubiquitinated protein but that tubulin-associated Las1 is not ubiquitinated.

**Ubiquitinated Las1 is a target for proteasomal destruction.**
As polyubiquitination generally targets proteins for proteasome-mediated destruction, we then determined the effect of the proteasome inhibitor MG132 (26) on the degradation of ubiquitinated Las1. COS-7 cells were transiently transfected with vectors encoding Las1-A/J and Las1-B6 and incubated in the presence or absence of MG132. We observed that addition of MG132 to cells significantly increased recovery of ubiquitinated Las1-A/J and Las1-B6 compared with the samples from mock (DMSO)-treated cells (Fig. 3B), suggesting that the proteasomal activity is required for ubiquitinated Las1 degradation. We also did similar assays in LM2 and E10 cells and got similar results (Supplementary Fig. S5), confirming that endogenous Las1 is polyubiquitin modified. Taken together, these results show that the degradation of Las1 is mediated by the 26S proteasome.

**Las1-A/J is a more stable protein.** We frequently observed larger amounts of Las1-A/J than Las1-B6 by transfecting cells with the same amounts of Las1-A/J and Las1-B6, suggesting that Las1-B6 degrades faster. To confirm this, COS-7 cells were transiently transfected with Las1-A/J and Las1-B6 along with or without pcDNAs-Flag-ubiquitin. The steady-state levels of Las1-A/J, when overproducing Flag-ubiquitin, were higher than Las1-B6 (Fig. 3C), suggesting that Las1-A/J is a more stable protein than Las1-B6. There were no obvious differences in the steady-state levels between Las1-A/J and Las1-B6 in the cells without co-over-expressing ubiquitin. We believe it is because of signal saturation. To further study the differential stabilities of Las1-A/J and Las1-B6, we determined the half-life of Las1-A/J and Las1-B6. As shown in Fig. 3D, inhibition of protein biosynthesis resulted in the loss of half the amount of total Las1-B6 within 2 h, whereas Las1-A/J was more stable with an approximate half-life of 6 h. These data agree with the above observations for Las1-A/J is degraded slower than Las1-B6.

**Las1 undergoes cell cycle–dependent expression.** Destruction of Las1 through the ubiquitin-proteasome pathway implies a potential regulation of Las1 expression during the cell cycle. To examine it, we did cell cycle analysis using E10-3 cells. As shown in Fig. 4A, Las1 fluctuated throughout the cell cycle. Las1 was not detected in G0 cells, started to accumulate in early S phase, reached the highest level in the G2 phase, and dropped to very low levels at mitosis. We also did similar experiments in E10 cells to determine whether the endogenous Las1 behaved similarly and got similar results (Supplementary Fig. S6). These results show that expression of Las1 is under cell cycle control.

Anti-Las1 immunoblotting revealed two Las1-B6 bands (Fig. 4A). Along with the fact that Las1-B6 carries Ser60 and degrades faster, this suggests that Las1 may get phosphorylated on Ser60 and recognized by a specific E3 ligase. To confirm this, we generated Las1-Asp60 (mimicking constitutive phosphorylation) and Las1-Ala60 (blocking phosphorylation) mutants and determined their steady-state levels. Las1-Asp60 displayed a lower steady-state level than Las1-B6, but Las1-Ala60 did not show a higher steady-state level even than Las1-B6 (Supplementary Fig. S7). These results suggest that conformational changes, and not phosphorylation at Ser60, account for the differential stabilities between Las1-A/J and Las1-B6.

**Ubiquitin-proteasome–mediated Las1 degradation occurs in mitosis.** To determine whether the ubiquitin-proteasome system is responsible for the quick degradation of Las1 in mitosis, E10-3 cells in exponential growth were synchronized in G2 and mitosis. We also did similar experiments in E10 cells to determine whether the endogenous Las1 behaved similarly and got similar results (Supplementary Fig. S6). These results show that expression of Las1 is under cell cycle control.

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indicating that Las1 ubiquitination does not occur in the G2 phase. However, upon MG132 treatment, high molecular weight forms of Las1 accumulated compared with Las1 from untreated mitotic-synchronized cells, indicating that destruction of Las1 in mitosis is mediated by the ubiquitin-proteasome system.

**Overproducing Las1-A/J causes defective cytokinesis in E10 cells.** We also generated E10-2 cells, a stable Las1-A/J cell line in which Las1-A/J is overexpressed in E10 cells. E10 and E10-3 cells, when cultured, reached confluence at a similar pace, but it took much longer time for E10-2 cells. To follow this, we seeded $8 \times 10^4$ of E10, E10-2, and E10-3 cells into 100-mm plates. At day 5, E10 and E10-3 cells reached much higher density than E10-2 cells (Supplementary Fig. S8). In addition to having less cell numbers, E10-2 cells also displayed some morphologic changes from mostly a refractive nucleus with thin and round cytoplasm to few a flat, round, and enlarged morphology (Fig. 5A). Some E10-2 cells showed two refractive nuclear areas with close contact to each other and surrounded with cytoplasm (Fig. 5A, bottom). Anti-β-tubulin and DAPI staining of the E0-2 cells confirmed that these cells harbored two nuclei (Fig. 5B). We therefore examined >300 cells of E10, E10-2, and E10-3 at days 2 and 5. Less than 1% of E10 and E10-3 cells were binucleated compared with 9% (day 2) and 26% (day 5) for E10-2 cells (Fig. 5C). These results suggest a cytokinesis defect in E10-2 cells introduced by overproducing Las1-A/J. We also observed some shrunken dumbbell-shaped cells that were loosely attached and floating in the medium, suggesting that the double-nucleated E10-2 cells died eventually.

**Discussion**

Aberrant regulation of the cell cycle is one mechanism that leads to cancer. To understand how Las1 contributes to lung cancer, we investigate whether Las1 has characters of the cell cycle regulatory proteins. Our goal was to study the cell cycle–dependent expression of Las1 in a normal cell line. E10 is an immortalized normal lung epithelial cell line from BALB/c mice and has relative complete set of cell cycle control system (27). We chose E10 as a parental cell line and generated E10-3 for Las1 cell cycle analysis. Our data, consistent between the overexpressed and endogenous levels, revealed a clear cell cycle–dependent expression pattern of Las1 peaking in G2 and declining in mitosis. Finding that ubiquitin-proteasome mediated Las1 degradation in mitosis further strengthens the notion for Las1 being a cell cycle regulator. Our data suggest that Las1-A/J is a more stable protein than Las1-B6. Slower degradation of Las1-A/J in mitosis may disturb some normal processes in after mitosis if Las1 plays a crucial role in cell cycle regulation. For example, ubiquitin-proteasome–mediated degradation of cyclin B is essential for progression of metaphase to anaphase. Blocking the degradation of cyclin B thus arrests cell cycle in metaphase (28). As predicted, E10-2 cells grew much more slowly than E10 and E10-3 cells. Light microscopy further revealed that many E10-2 cells (10–30%) are binucleated during exponential cell growth, suggesting a cytokinesis defect. These findings further prove that Las1 is a crucial cell cycle regulator.

We identified tubulin as Las1-interacting protein and further showed that Las1 associates with microtubules. Las1-A/J and Las1-B6 differ in one residue but display differential stabilities toward ubiquitin-proteasome–mediated degradation. However, our data suggest that there are no differences between Las1-A/J and Las1-B6 in binding to tubulin. We have further mapped the tubulin-binding domain of Las1 to residues 195-381, therefore further showing that residue 60 that differs between Las1-A/J and Las1-B6 is not required for tubulin binding. We show that the portion of Las1 associated with tubulin is not ubiquitinated. This result suggests that the association with microtubule and ubiquitination/degradation of Las1 happen sequentially with dissociation of Las1 from microtubules first. Expression in G2 and colocalization with microtubules in interphase suggests that the interaction of Las1 and microtubules likely occurs in the G2 phase. We have tried to determine whether Las1 binding stabilizes microtubules by treating cells with various concentrations of nocodazole but did not observe anything. Currently, the functional significance of the association of Las1 with microtubules remains unclear. The interaction of Las1 and tubulin may mediate important biological events. However, it may not be the reason for the differential susceptibilities of A/J and C57BL/6j mice to lung tumorigenesis because Las1-A/J and Las1-B6 bind to tubulin with equal affinity.

Differential degradation of Las1-A/J and Las1-B6 leads to opposite consequences in normal and tumor cells. As summarized in Fig. 6A, degradation of Las1 is required for cell cycle progression in normal cells, whereas it slows down proliferation in human (our recent study; data not shown) and mouse tumor cells (4). Taken together, our results lead to a working model in which the degradation of Las1 in tumor cells leads to activation of a yet unidentified downstream signaling pathway (X) that inhibits tumor cell growth, whereas this pathway is inactive ($\chi$) in normal cells (Fig. 6B). Identification and characterization of this pathway will further decipher the role of Las1 in lung tumorigenesis.

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**References**

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