4

LRSAM1 E3 Ubiquitin Ligase Promotes Proteasomal Clearance of UBE3A/E6-AP

Previous research demonstrates that LRSAM1 contributes in the development of the brain, spinal cord and tail formation in *Drosophila melanogaster* and a knockdown of LRSAM1 disturbs the formation of these organs [Weterman *et al.*, 2012]. Other than neurodevelopment, there is increasing evidence of LRSAM1 involvement in different neurodegenerative disorders. Hence, LRSAM1 has implications in both, i.e., neurodevelopmental and neurodegenerative disorders. Thus, LRSAM1 can participate in the pathology of neurodevelopment or neurodegeneration possibly by modulating proteins, which are observed to regulate both these mechanisms. Here, it was found that LRSAM1 is involved in the degradation of E6-AP and overall affected its turnover including the basal levels of E6-AP critical substrates: tumour suppressor protein wild-type p53 and p27. LRSAM1 mediated E6-AP degradation is may be associated with the consequences of neurodevelopment or neurodegenerative conditions; especially it can be linked with neurodevelopmental disorder like Angelman Syndrome, where E6-AP functions are lost. The study also indicates the presence of a cellular strategy where LRSAM1 may control the basal level of QC protein, i.e. E6-AP, which in stress conditions is observed to be induced and provides cytoprotective effects. A co-localization and clear recruitment of LRSAM1 with E6-AP protein are present in various stresses, and LRSAM1 can induce the endogenous turnover of E6-AP. Partial depletion of LRSAM1 elevates the levels of E6-AP and affect overall cell cycle regulatory proteins (p53 and p27) expression, including the rate of cellular proliferation.

4.1 RESULTS

LRSAM1 induced proteasomal degradation of E6-AP is demonstrated by many different experiments and is explained in the below-given figures.

4.1.1 Under Cellular Stresses Conditions Both LRSAM1 And E6-AP Are Recruited As Inclusion Bodies Like Structures Towards Nuclear Peripheral Region

Cells always try to remove unwanted, old and aberrant proteins with the help of PQC machinery. Lack of PQC function in the removal of misfolded proteins may cause development of insoluble aggresomes bodies in the cells, which can lead to various cellular metabolic defects. Few selective E3 ubiquitin ligases function as QC regulators and thus can mark the misfolded aberrant proteins for removal from the cells. Lack of LRSAM1 E3 ubiquitin ligase function cause axons of the peripheral region to degenerate in a mice animal prototype of CMT 2P disease. However, the functions of LRSAM1 in the maintenance of proteostasis with the help of PQC are elusive and knowledge of the same may help to better elucidate LRSAM1 function in CMT 2P. Still, it is not well understood, how LRSAM1 can have an important role in early cellular protection mechanism against aggregation-mediated proteotoxicity under various cellular stresses? To investigate this query, cells were cultured into the 2-well chamber slides and given different cellular stress inducing conditions. During one set of experiment, cells were also given heat-treatment (43°C, 30 minutes) and afterward control and treated cells were examined by immunofluorescence imaging using anti-LRSAM1 and anti-E6-AP antibody (Figure 4.1).

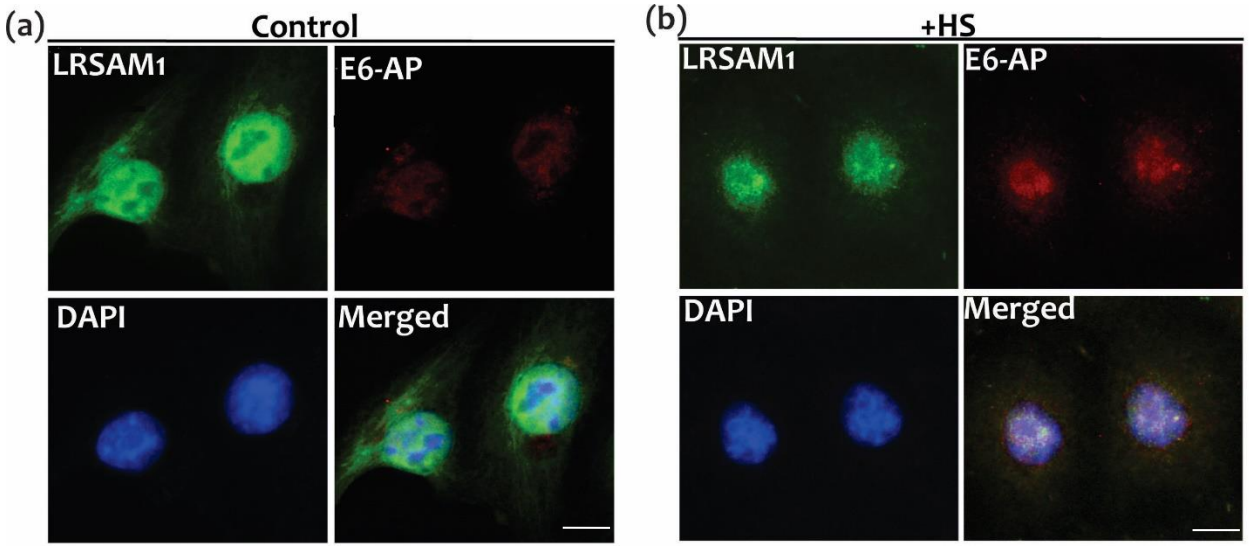

Figure 4.1 : *LRSAM1 and E6-AP were up-regulated in reply to heat stress:* (a-b) Cos-7 cells cultured using 2-well chamber slides were given heat stress (HS) of (43°C,30 minutes) and then used for immunofluorescence microscopy with LRSAM1 (green) and E6-AP (red) antibodies. Nuclei of the cells were labelled with DAPI stain. Scale Bar: 20 µm. **Manuscript submitted for publication (under review) as Ribhav Mishra and Amit Mishra et al., 2019.**

Since previous findings suggest E6-AP act as a cellular QC E3 ubiquitin ligase and target misfolded proteins for their elimination with the assistance of molecular chaperone Hsp70 [Mishra *et al.*, 2009]. In the current study, we surprisingly found that LRSAM1 E3 ubiquitin ligase under various types of stresses follows the similar expression profile of E6-AP i.e., a prominent expression of LRSAM1 occurred in the nuclear peripheral region compared to cytoplasmic compartment (Figure 4.2 (a-b)). Fluorescence micrograph analysis suggests that under autophagic dysfunction (25 µM , 12 hr) (Figure 4.2 (a)) and proteasomal stress (10 µM, 12 hr) (Figure 4.2 (b)) conditions, LRSAM1 protein gets predominately accumulated to nuclear peripheral region as inclusion bodies and E6-AP also follows the similar clear big aggresomes like structures. Overlay images with DAPI staining clearly indicate their co-localization in the form of inclusions like arrangement in the response to different cellular stress conditions.

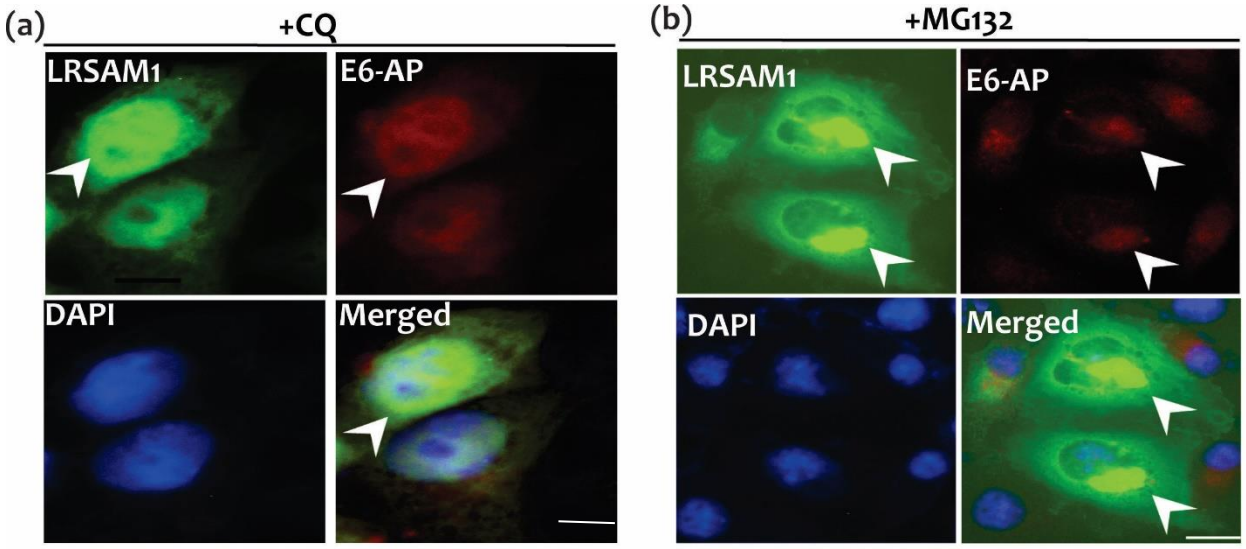

Figure 4.2 : *Recruitment of LRSAM1 and E6-AP to nuclear peripheral region in conditions of proteotoxic stress: (a-b)* Cos-7 cells were given doses of chloroquine (CQ) (25 µM, 12 hr) and MG132 (10 µM, 12 hr) and then examined using immunofluorescence microscopy with antibodies of LRSAM1 (green) and E6-AP (red).

Arrowheads denote LRSAM1 and E6-AP co-localization to the nuclear peripheral region. The nucleus was DAPI labelled. Scale bar: 20 μm.

These preliminary results suggest that LRSAM1 may participate in the cellular PQC pathway and can follow the function profile of other QC E3 ubiquitin ligases such as E6-AP involved in stress-related conditions.

4.1.2 *E3 Ubiquitin Ligase LRSAM1 Overexpression Reduces the Endogenous Levels of E6-AP Protein*

To examine whether the subcellular co-localization of LRSAM1 with E6-AP affects its molecular functions, cells expressing wild type (LRSAM1) and mutant (LRSAM1ΔRING) forms were used, and their exogenous overexpression was established by immunoblotting using anti-Myc antibody (Figure 4.3 (a)). Same sample sets were also used to probe the endogenous amounts of other QC E3 ubiquitin ligases (E6-AP or CHIP). Interestingly it was observed that endogenous levels of E6-AP protein dramatically reduces under LRSAM1 overexpression and the level of CHIP was not affected in the same sample set as shown in Figure 4.3 (b). These observations encouraged us to further examine the precise effect of LRSAM1 mutant on E6-AP protein. Therefore we used mutant (LRSAM1ΔRING) sets of samples for detailed immunoblot analysis and observed that mutant or defective LRSAM1ΔRING catalytically inactive form overexpression does not affect the endogenous amounts of both QC E3 ubiquitin ligases, i.e. E6-AP or CHIP, as revealed by Figure 4.3 (c).

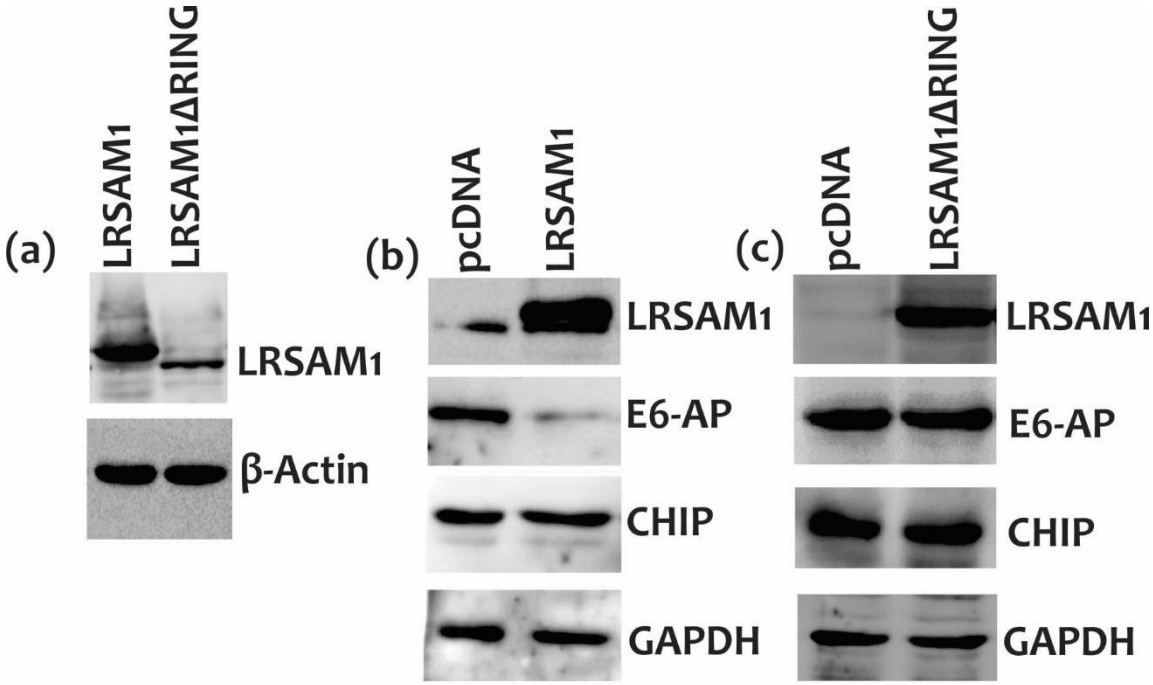

Figure 4.3 *: LRSAM1 exogenous transfection modulates the level of E6-AP in the cell:* (a) LRSAM1-Myc and LRSAM1ΔRING overexpressing cell lysates of Cos-7 cell were immunoblotted using antibodies of Myc and β-actin. (b) Cos-7 cells overexpressing control or LRSAM1 were examined using antibodies of LRSAM1, E6-AP, CHIP, and GAPDH. (c) Similar to (b) cell lysates of Cos-7 cells expressing control or LRSAM1ΔRING were also evaluated.

The preliminary results suggest that LRSAM1 and E6-AP co-localizes under stress conditions and overexpression of LRSAM1 partially depletes the level of E6-AP protein in cells and not its catalytically inactive form (LRSAM1ΔRING). Therefore, we wanted to check the concentration-dependent effects of LRSAM1 on the E6-AP E3 ubiquitin ligase. To further ascertain that the active LRSAM1 (Figure 4.4 (a)) was overexpressed in an increasing

concentration in the cells, the collected lysate was examined by western blotting analysis. As represented in Figure 4.4 (a-b) overexpression of LRSAM1 in an increasing concentration successively reduces the levels of E6-AP protein in cells.

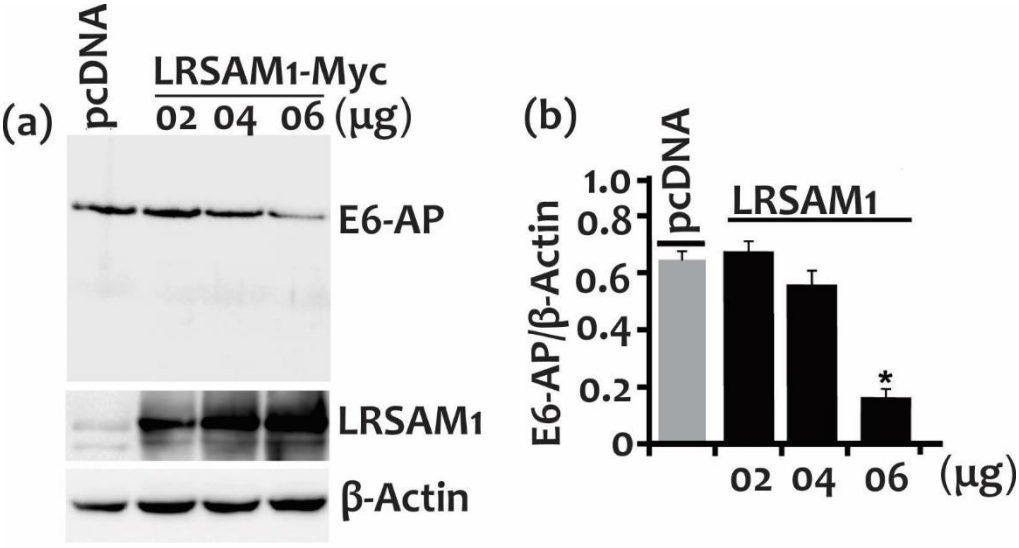

Figure 4.4 : *LRSAM1 overexpression in a concentration dependent manner degrades E6-AP:* (a-b) LRSAM1-Myc was overexpressed in an increasing concentration in Cos-7 cells, and the cell lysates of the same were collected and immunoblotted with antibodies of E6-AP, LRSAM1, and β-actin. The band intensity of E6-AP/β-actin was quantified from these blots and is given in (b), and the values shown are derived from three individual experiments and is estimated from mean±SD *p<0.05 for LRSAM1 transfected cells in comparison to control.

However, a concentration-dependent overexpression of an inactive form of LRSAM1ΔRING was not effective in degrading the endogenous levels of E6-AP protein, as shown in Figure 4.5 (a-b).

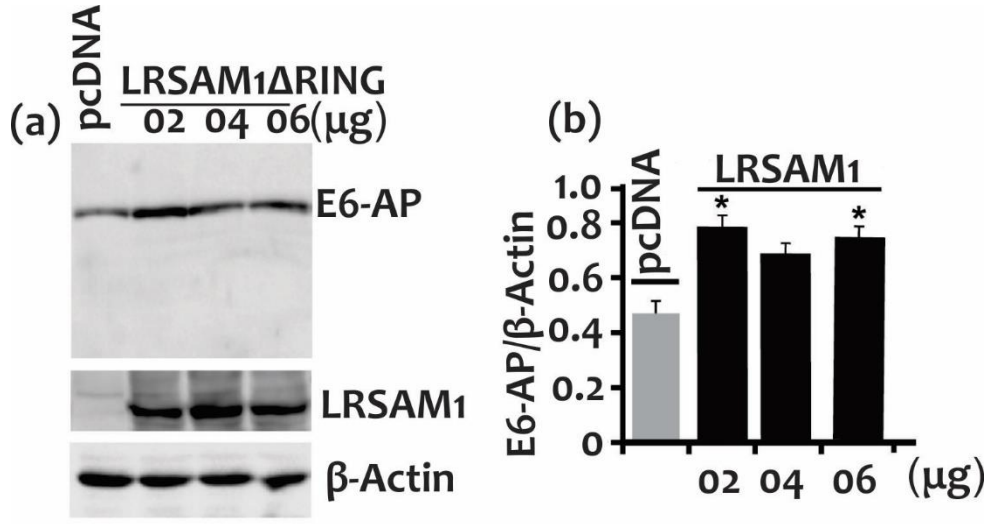

Figure 4.5 : *Overexpression of LRSAM1ΔRING do not affect E6-AP level:* (a) LRSAM1ΔRING was transfected in Cos-7 cells in an increasing concentration. Lysates of the above cells were immunoblotted by different antibodies of E6-AP, LRSAM1, and β-actin. (b) The band intensity of E6-AP/β-actin from (a) was quantified. The values presented are derived from mean±SD and where*p<0.05 for LRSAM1ΔRING transfected cells in comparison to pcDNA transfected cells.

Our results shows it may be possible that LRSAM1 specifically recognizes E6-AP protein for their removal from the thick cellular pool, yet for the better cellular physiological understanding of the molecular mechanism involved in this process in near future more observation in detail is essential.

4.1.3 LRSAM1 Overexpressed Cells Retain Depleted Amounts of E6-AP QC E3 ubiquitin Ligase

As previous findings suggested that under stress situations, cellular PQC mechanism governs the clearance of massive accumulation of aberrant proteins most probably by up-regulation of different types of chaperones and QC E3 ubiquitin ligases. But it is essential to understand that after coping up against the cellular stress conditions with efficient PQC functions, later how cells solve the challenges of the overburden production of QC E3 ubiquitin ligases and chaperones. Previously, it has been shown that CHIP not only promotes Hsp70 induction under stress condition but also regulates Hsp70 under stress conditions [Qian *et al.*, 2006]. Interestingly, CHIP also induces the stabilization of aggregate-prone Malin protein via Hsp70 activity modulation in cells [Rao *et al.*, 2010]. Most likely, LRSAM1 can also preferentially target E6-AP for degradation during stress recovery conditions when aberrant misfolded proteins have been eliminated from the dense cellular pool. Therefore it was vital for us to observe how LRSAM1 is significant in the regulation of QC E3 ubiquitin ligase E6-AP. To answer this question, we have transiently transfected LRSAM1 construct in concentration-dependent manner and validated LRSAM1 overexpression using RT-PCR, immunoblotting and immunocytochemistry experiments (Figure 4.6 (a-c)).

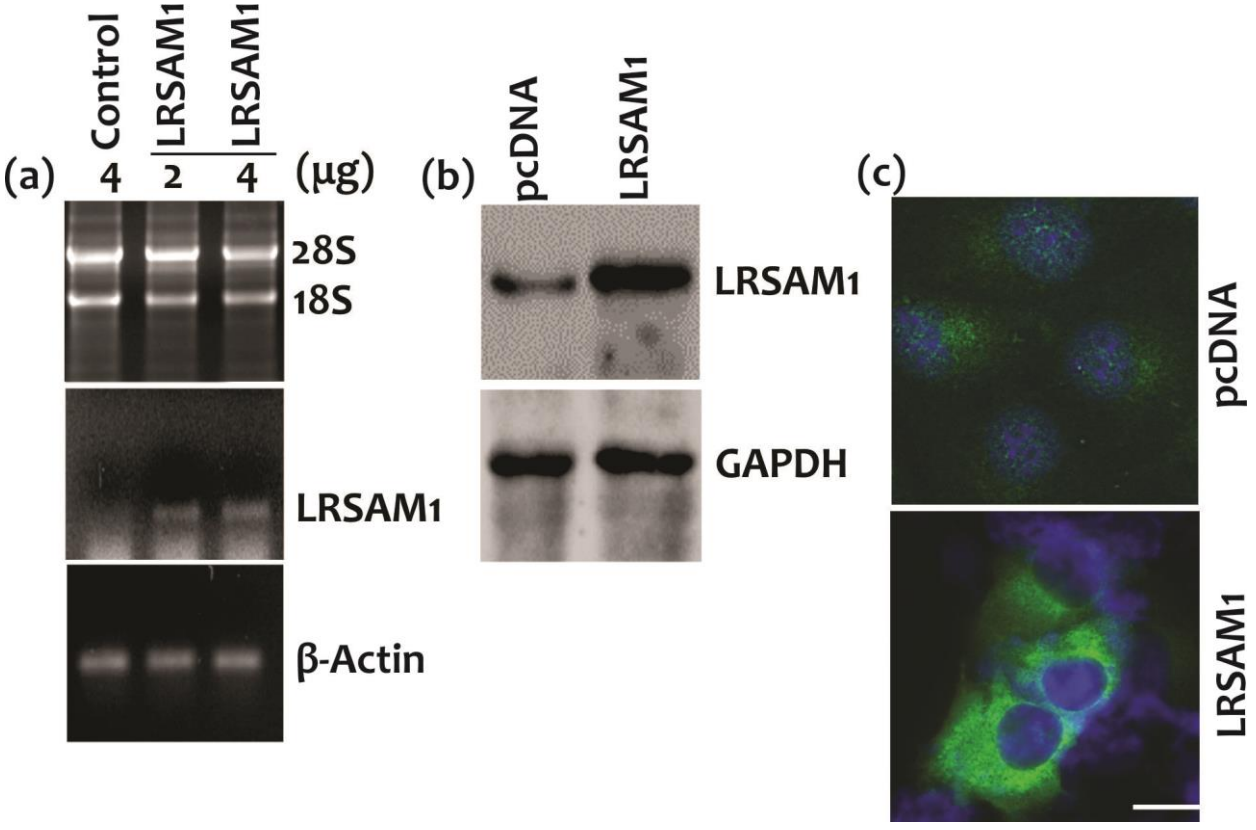

Figure 4.6 : *LRSAM1 overexpression analysis:* (a) A549 cells were overexpressed with LRSAM1-Myc in an increasing concentration. RNA from the above-prepared cells were isolated and is used for RT-PCR with the help of LRSAM1 and β-actin specific primers. (b) In immunoblotting, Cos-7 cell lysates overexpressing pcDNA or RSAM1-Myc plasmid were probed with LRSAM1 and GAPDH antibodies. (c) Immunocytochemistry of LRSAM1-Myc and pcDNA overexpressing Cos-7 cells were performed with the help of LRSAM1 specific antibodies. DAPI was used to label the nucleus. Scale bar: 20 µm.

In different experiments, Cos-7 cells overexpressing pcDNA-EGFP or LRSAM1-Myc or LRSAM1ΔRING were immunostained with c-Myc and E6-AP. Immunofluorescence micrographs analysis reveal overexpression of LRSAM1 reduces the level of E6-AP protein in cells (Figure 4.7 (b)) compared to control (pcDNA-GFP) transfected cells (Figure 4.7 (a)). Previously, it has been reported that an accumulation of misfolded proteins sequesters various useful proteins and severely affects normal cellular functions. Therefore it became crucial for us to understand how mutant form of LRSAM1ΔRING co-localizes with E6-AP. Immunofluorescence analysis of overexpressed LRSAM1ΔRING cells probed with anti-Myc antibody demonstrates that the over accumulated mutant LRSAM1ΔRING co-localizes with E6-AP in cells with no change in expression of E6-AP (Figure 4.7 (c)).

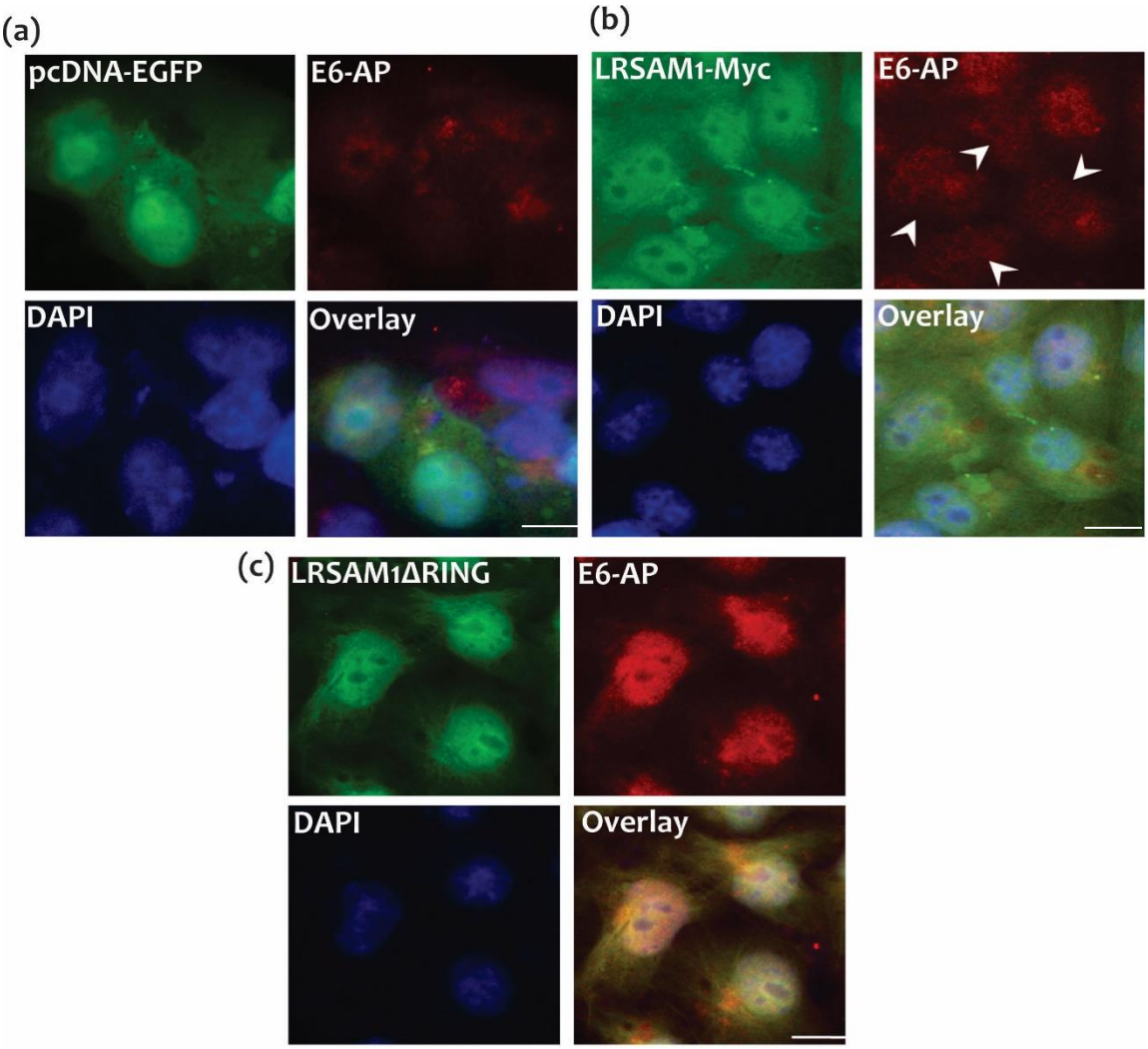

Figure 4.7 : *LRAM1-Myc overexpression induces E6-AP elimination with no effect in LRSAM1 ΔRING:* (a-c) Cos-7 cells overexpressing pcDNA-EGFP or LRSAM1-Myc or LRSAM1ΔRING were used for immunostaining with the help of c-Myc and E6-AP antibodies. The arrowheads indicate E6-AP depletion in LRSAM1 overexpressing cells. Nucleus was labelled using DAPI and Scale Bar: 20 µm.

These observations suggests that only an active form of LRSAM1 is efficient in reducing the level of E6 AP protein and may stimulate the removal of E6-AP from the cellular environment.

4.1.4 LRSAM1 Overexpression Degrades E6-AP and Accumulates E6-AP Substrates (Putative Cell Cycle Proteins) Which Downregulates Cellular Proliferation

As we observed that LRSAM1 induces the elimination of E6-AP, we wondered whether LRSAM1 overexpression could affect the E6-AP substrates dependent cellular physiological

functions. Therefore, we transfected LRSAM1 in cells, and collected samples were processed for detailed immunoblot analysis. As demonstrated in Figure 4.8 (a), overexpression of LRSAM1 reduces the E6-AP protein and induces the accumulation of E3 ubiquitin ligase E6-AP substrates such as p53 and p27. We confirmed LRSAM1 overexpression with the help of immunblotting performed using anti-LRSAM1 antibody (Figure 4.8 (b).

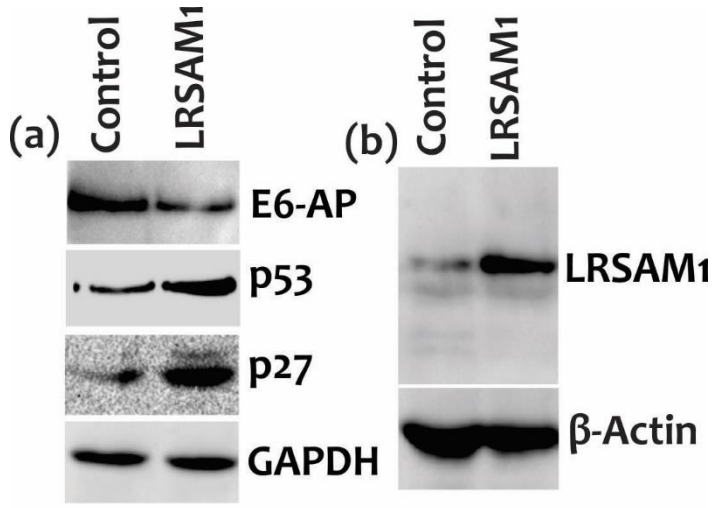

Figure 4.8 : *LRSAM1 mediated degradation of E6-AP causes up-regulation of its cell cycle substrate proteins p53 and p27:* (a) Cos-7 cells overexpressing control or LRSAM1-Myc were immunoblotted with E6-AP, p53, and p27 proteins. (b) The same set of prepared cells were also checked for LRSAM1 expression using LRSAM1 antibody.

Cos-7 cell overexpressed in an increasing concentration with LRSAM1 were used for immunofluorescence examination using LRSAM1 (green) and E6-AP (red) antibodies, as shown in Figure 4.9. Some of the same sets of cells were given doses of MG132. The results indicate that the E6-AP endogenous level was depleted in those cells, which overexpresses LRSAM1 and the influence of LRSAM1 on E6-AP protein can be reversed with the addition of putative proteasomal inhibitor (MG132) in cells.

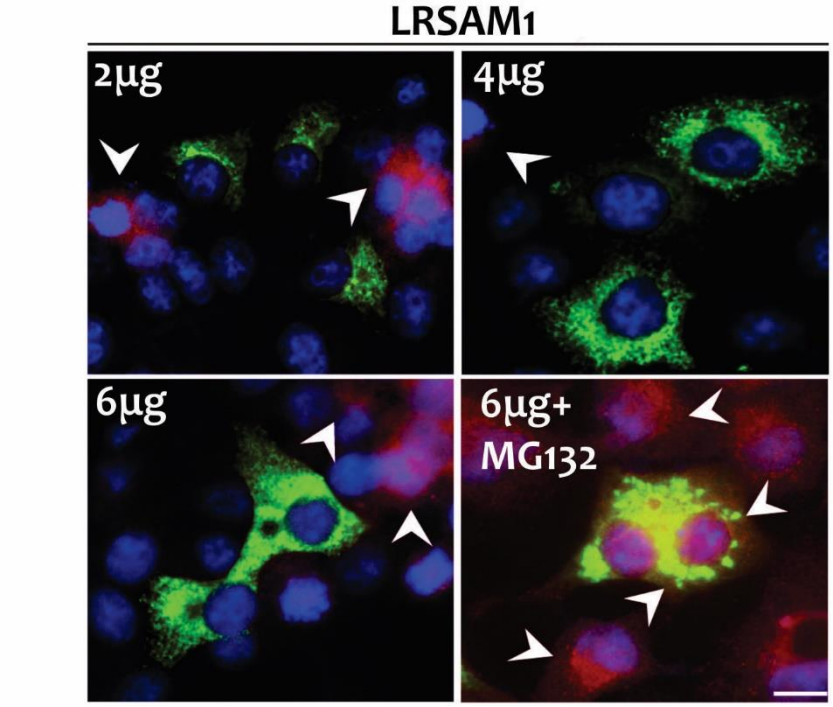

Figure 4.9 : *LRSAM1 induces proteasome dependent degradation of E6-AP: Cos-7 cells expressing LRSAM1 in a increasing concentration were stained with LRSAM1 (green) and E6-AP (red) antibodies. Few of the same set of cells were also exposed to MG132* and then detected with LRSAM1 antibody. Immunofluorescence micrographs of the cells were acquired using fluorescence microscopy and nuclear staining was performed with the help of DAPI. Arrowheads show depletion of E6-AP in LRSAM1 overexpressing cells. Scale bar 20 µm.

Our previous findings suggest that E6-AP targets p53 and p27 for proteasomal degradation [Mishra *et al.*, 2009, Mishra and Jana, 2008]. Here we have noticed that LRSAM1 targets E6-AP for the intracellular degradation and since E6-AP substrates, i.e. p53 and p27 are cell cycle regulators; therefore consequently it was crucial for us to validate the effect of E6-AP depletion on overall cellular proliferation. Bright field microscopic images clearly indicate that overexpression of LRSAM1 in time-dependent manner reduces the rate of cellular proliferation, as shown in Figure 4.10. These findings suggest most likely that LRSAM1 mediated depletion of E6-AP protein induces the accumulation of its critical substrates proteins like p53 and p27, which may further lead to the suppression of cellular proliferation.

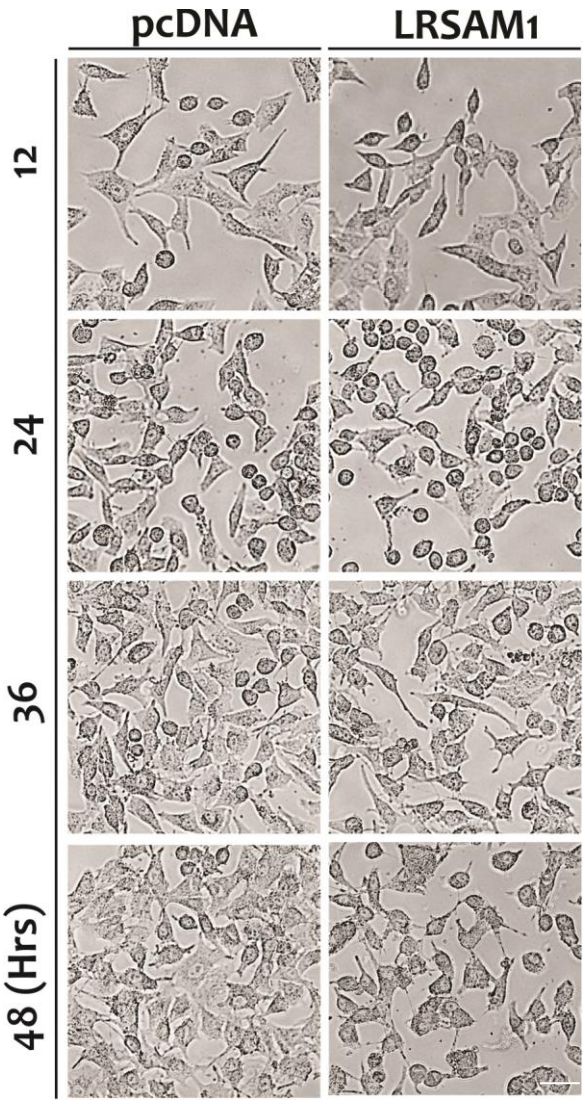

Figure 4.10 : *Overexpression of LRSAM1 modulates cellular proliferation:* A549 cells were overexpressed either with pcDNA or LRSAM1-Myc and then observed with the help of bright field imaging at different intervals of 12, 24, 36, and 48 hrs. Scale Bar: 20 20 µm.

Overall lack of E6-AP can lead to the cell cycle suppression and can also induce apoptosis, however in the near future, the notion that LRSAM1 mediated E6-AP regulation can up-regulate apoptosis needs further detail study for our better understanding.

4.1.5 Partial Depletion of LRSAM1 Stabilizes Intracellular E6-AP Protein

To address whether there is a direct functional regulation of E6-AP mediated by the expression of LRSAM1, we performed siRNA based knockdown analysis, as shown in Figure 4.11. A549 cells propagated in the 6-well tissue culture plates were exogenously expressed with siRNA oligonucleotides directed against LRSAM1 or control (scrambled siRNA). Post-transfected cells after 24 hr were used for RNA collection and RT-PCR evaluation, as shown in Figure 4.11 (a-b). RT-PCR findings confirm a partial knockdown of LRSAM1 in cells.

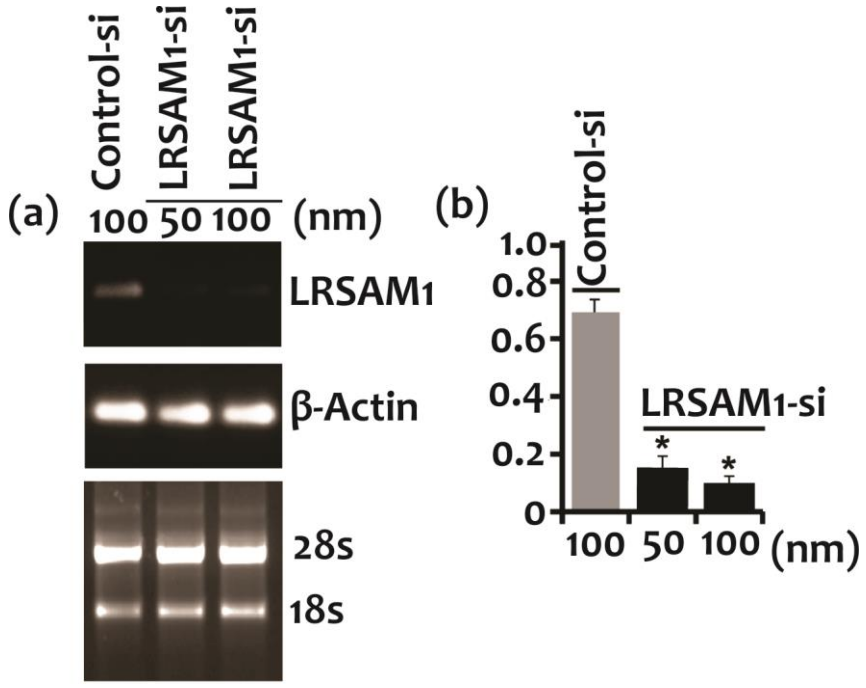

Figure 4.11 : *RT-PCR analysis of LRSAM1 knockdown samples in an increasing concentration:* (a) Control-si or LRSAM1-si were transfected in A549 cells in a concentration dependent manner. The RNA from the cells were isolated and shown. The RNA was used for RT-PCR analysis with the primers of LRSAM1 and β-actin. (b) Band intensities of LRSAM1 was quantified from (a), and the given values are a measure of mean±SD and *p<0.05 for LRSAM1-si samples.

Control-si and LRSAM1-si expressing A549 cells, as prepare for RT-PCR analysis were also immunoblotted using the antibodies of LRSAM1, E6-AP, and β-actin. The finding from the immunoblots indicates accumulation of E6-AP in LRSAM1-si expressing cells in comparison to control-si transfected cells (Figure 4.12 (a-b)).

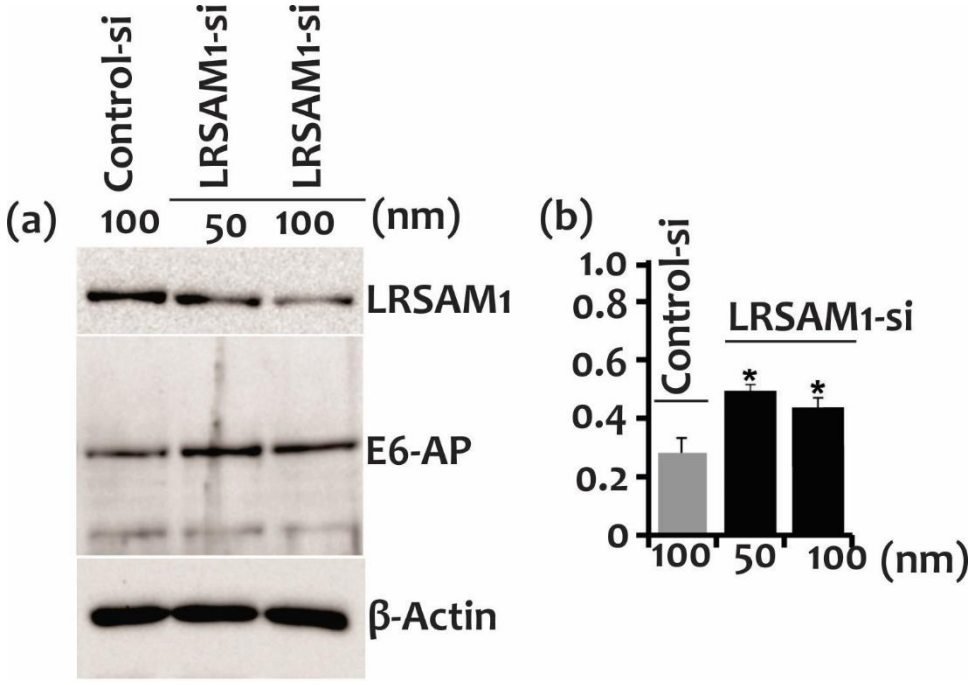

Figure 4.12 : *LRSAM1 knockdown in an increasing concentration causes upregulation of E6-AP protein:* (a) Control-si and LRSAM1-si oligonucleotides were used for transfecting A549 cells. The cells lysates of the cells were then immunoblotted using antibodies of LRSAM1, E6-AP and β-actin. (b) Band intensities of E6-AP/β-actin

were quantified by estimating the mean±SD value and *p<0.05 for LRSAM1-si transfected cells in comparison to control-si expressing cells.

We have also confirmed the above immunoblot observation with the help of immunofluorescence analysis in LRSAM1 depleted cells. As shown in Figure 4.13, partial knockdown of LRSAM1 exhibited marginal accumulation of E6-AP in cells.

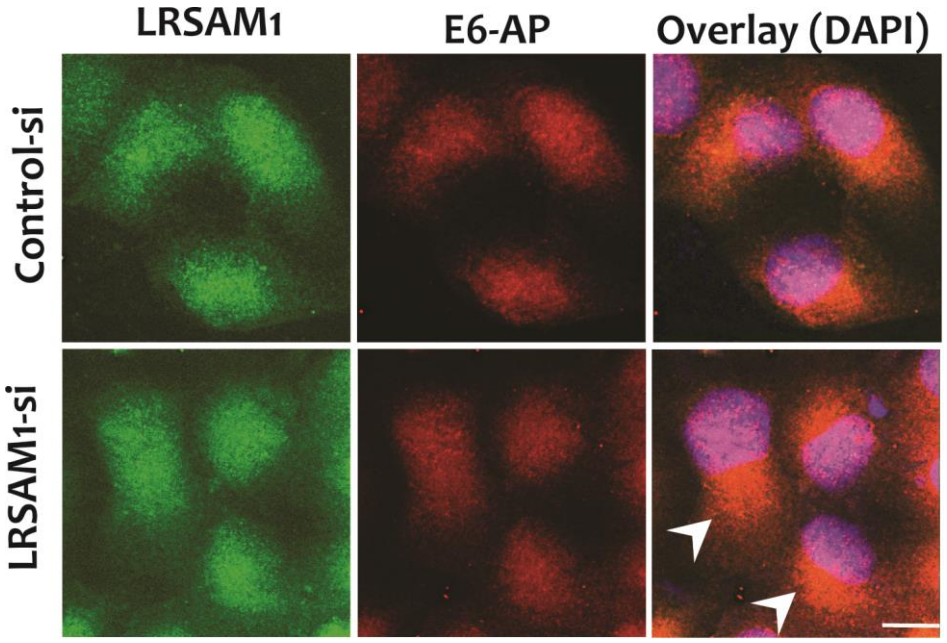

Figure 4.13 : *Immunocytochemistry of LRSAM1-knockdown cells*: Control-si or LRSAM1-si were transfected in A549 cells and the post-transfected cells were examined using LRSAM1 (green) and E6-AP (red) antibodies via immunocytochemistry. Cell nuclei were stained by DAPI. Arrowheads indicate E6-AP marginal accumulation in LRSAM1-si expressing cells compared to control. Scale bar 20 μm.

Largely these observations emphasize that LRSAM1 is might be involved in modulating E6-AP levels in cells and thus possibly participate in the clearance of excess amount of E6-AP protein elevated during the recovery phase of cells against cellular stress conditions.

4.1.6 LRSAM1 Partial Knockdown Accumulates E6-AP and Modulates Cellular Proliferation Rate

In the previous results, it was observed that the overexpression of LRSAM1 reduces E6-AP, decreases cellular division, and partial knockdown of LRSAM1 causes intracellular accumulation of E6-AP. Induced amount of p27 and p53 in E6-AP depleted cells can play important role in the modulation of cellular proliferation responses. Hence, in order to better understand these findings, it was essential for us to monitor the cellular proliferation rate in LRSAM1 depleted cells. Therefore, cells were expressed with siRNA oligonucleotides against LRSAM1 or control (scrambled siRNA) and collected lysates were evaluated by immunoblotting as illustrated by Figure 4.14 (a-b). The results suggest a knockdown of LRSAM1 promotes the accumulation of E6-AP. However, previous findings indicate that E6-AP is involved in the intracellular degradation of p27 and p53, so depletion of LRSAM1 and accumulation of E6-AP may alter p27 and p53 quantity in cell [Jiang *et al.*, 1998, Khan *et al.*, 2006, Mishra *et al.*, 2009, Mishra and Jana, 2008].

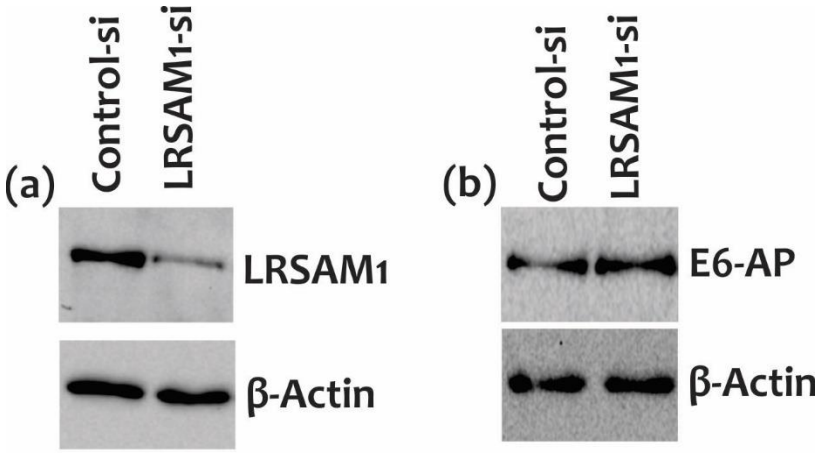

Figure 4.14 : *Effect of LRSAM1 knockdown on E6-AP accumulation by immunoblotting:* (a) A549 cells transfected with control-si or LRSAM1-si oligonucleotides were immunoblotted via the antibodies of LRSAM1, E6-AP, and β-actin.

LRSAM1-si and control-si expressing A549 cells were used in immunocytochemistry experiment performed with E6-AP antibody, and the result (Figure 4.15) indicates accumulation of E6-AP in LRSAM1-si transfected cells in comparison to control-si cells.

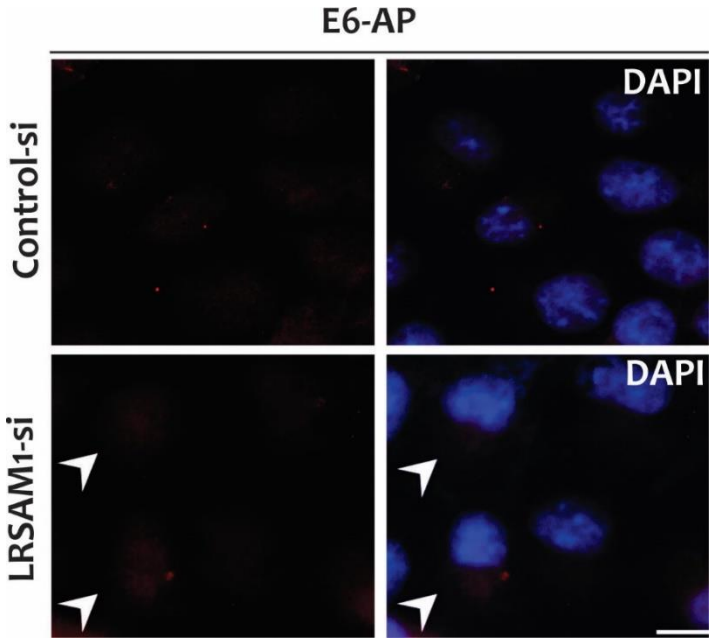

Figure 4.15 : *Analysis of LRSAM1 knockdown on E6-AP:* A549 cells transfected with control-si or LRSAM1-si were stained with E6-AP specific antibodies and then fluorescence micrographs of the cells were obtained with the help of immunofluorescence microscopy. Arrowheads denote accumulated E6-AP after LRSAM1 knockdown. Scale Bar: 20 μm.

In an attempt to examine the consequence of LRSAM1 knockdown on cellular proliferation; we have performed detailed bight field microscopic image examination for LRSAM1-si expressing cells. As shown in Figure 4.16, knockdown of LRSAM1 increase overall cellular proliferation, most likely, this could be the consequence of accumulated E6-AP and a resulting reduced amount of p53 and p27 in such cells.

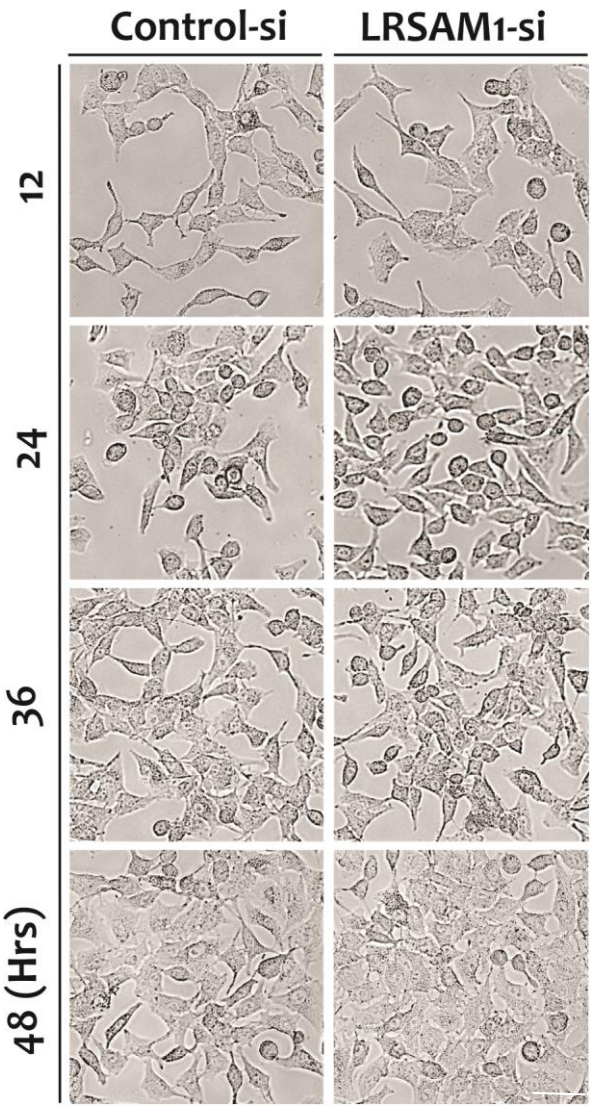

Figure 4.16 : *Knockdown of LRSAM1 modulates cellular division:* A549 cells expressed with LRSAM1-si or control-si were observed at different time durations using bright field microscopy. Scale Bar: 20 µm.

Taken together all these results shows that LRSAM1 may control E6-AP, which can cause an alteration in cell cycle progression by changing the levels of its cell cycle substrate proteins such as p53 and p27.

4.1.7 Expression of LRSAM1 Induces the Rate of Degradation of E6-AP

As we have shown that LRSAM1 targets E6-AP for intracellular degradation and also co-localize in the forms of inclusions with E6-AP under various stress conditions. We wonder if LRSAM1 could alter the rate of degradation or the half-life of E6-AP, therefore to answer this question, we have performed a cycloheximide chase experiment. Before executing the detailed cycloheximide chase experiment, we first assessed the overexpression profile of LRSAM1 by immunoblot analysis using Myc antibody to accurately detect exogenously expressed LRSAM1-Myc protein in cells (Figure 4.17 (a)). Next, we have plated cells into six-well plates and chased them with cycloheximide, after 24 hours of transfection. As presented in Figure 4.17 (b-c), overexpression of LRSAM1 induces the degradation of E6-AP with a much faster rate as compared to control, and treatment of MG132 (putative proteasome inhibitor) revert the effect of LRASM1 and reduces the rate of degradation. These results suggest that LRSAM1 recognizes E6-AP for proteasomal degradation and induces its turnover (Figure 4.17 (d)).

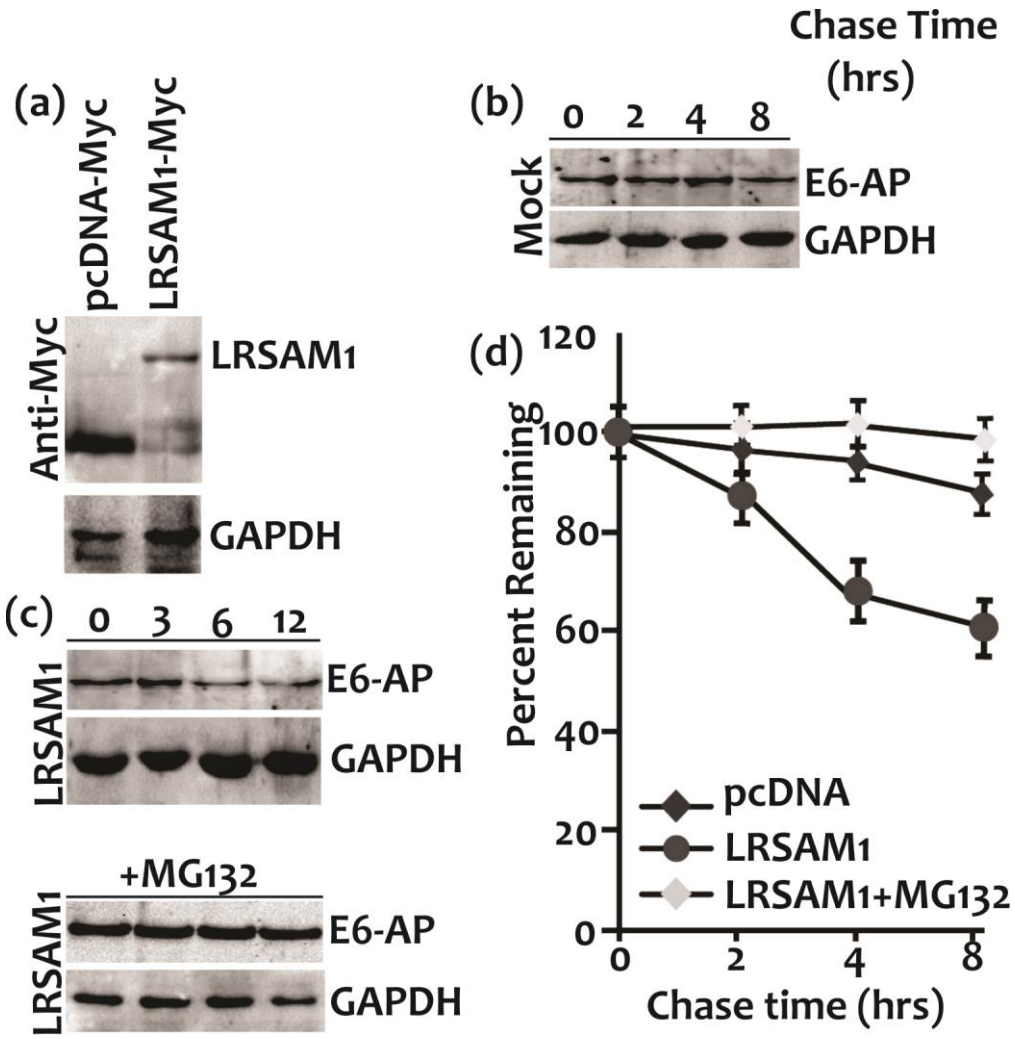

Figure 4.17 : *LRSAM1 modulates E6-AP protein turnover: (a)* Cells overexpressed with pcDNA-Myc or LRSAM1-Myc were detected with the antibodies of c-Myc and GAPDH. (b-c) Cos-7 cells overexpressed with mock or LRSAM1 were chased with cycloheximide (25 µg/ml) for several time durations. Some of the LRSAM1-Myc transfected cells were also exposed to MG132. The cell lysates of the above cells were then immunoblotted and analysed using antibodies of E6-AP and GAPDH. (d) The band intensities of mock, LRSAM1 and LRSAM1+MG132 transfected cells were prepared and is plotted as percent remaining vs. chase time. The given values are derived from mean±SD and *p<0.05 for LRSAM1 overexpressing cells as compared to pcDNA transfected cells.

As shown by the chase experiment, LRSAM1 overexpression leads to increase the turnover rate of E6-AP; therefore it was also crucial for us to further check the level of E6-AP in LRSAM1-Myc transfected cells by other experiments like immunocytochemistry. As illustrated in Figure 4.18 (a), E6-AP level is significantly reduced in the cells which overexpress LRSAM1 E3 ubiquitin ligase, and addition of MG132 revert the effect of LRSAM1 which lead to further accumulation of E6-AP in cells (Figure 4.18 (b)). In PQC pathway, E3 ubiquitin ligase determines the specificity in the marking and removal of the respective critical substrates and these catalytic activities of E3s provide functional efficiency in the degradation pathway associated with the molecular mechanisms of substrates recognition and degradation. Hence it was essential for us to check how the lack or aberrant function of LRSAM1 affects substrate degradation. We address this notion by overexpression of catalytically inactive LRSAM1ΔRING form in cells. As shown in Figure 4.18 (d), immunofluorescence analysis uncovers LRSAM1ΔRING mutant form exogenous expression is ineffective in E6-AP degradation. The levels of E6-AP protein remain unaltered in LRSAM1ΔRING post-transfected cells in comparison to control (pcDNA-GFP) cells (Figure 4.18 (c)).

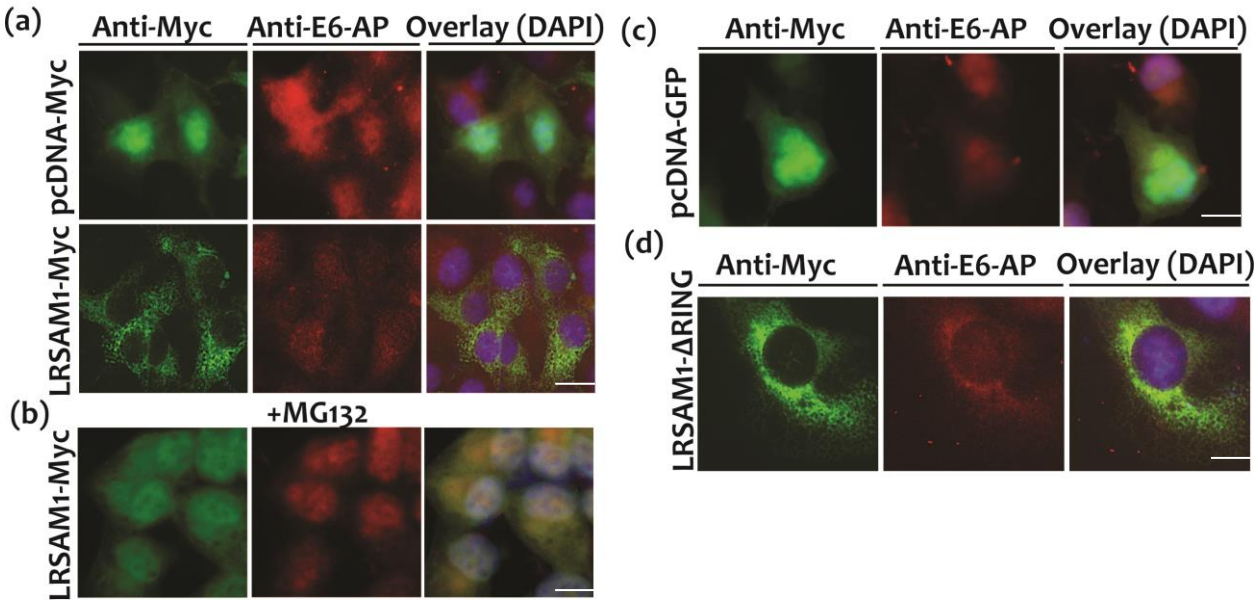

Figure 4.18 : Immunocytochemistry of effects of LRSAM1-Myc and LRSAM1ΔRING overexpression on E6-AP: (a-b) Cos-7 cells overexpressed with pcDNA-Myc or LRSAM1-Myc were labelled using immunofluorescence microscopy with the help of c-Myc (green) and E6-AP antibodies. Few of the LRSAM1-Myc transfected cells from above were given a treatment of MG132 and also used similarly for immunofluorescence examination as explained previously. (c-d) pcDNA-GFP or LRSAM1-ΔRING transfected cells were examined using immunocytochemistry with the help of LRSAM1 and E6-AP specific antibodies. DAPI was used for nucleus staining. Scale bar: 20 µm.

These findings demonstrate that LRSAM1 active form promotes the proteasomal degradation of E6-AP and modulate its turnover in cells.

4.1.8 LRSAM1 Knockdown Causes The Accumulation of E6-AP Degrades Cell Cycle Regulatory Proteins

To investigate the mechanism of proteasomal recognition of E6-AP by LRSAM1, the endogenous levels of LRSAM1 was knocked down by synthetic siRNA. Obstruction in the ubiquitin-dependent proteasome degradation of a critical substrate should result in its intracellular build-up and an increase in the endogenous amount of the substrate proteins. Therefore to answer this question in terms of E6-AP recognition mediated by LRSAM1 based proteasome degradation, we have depleted LRSAM1 by the use of LRSAM1-siRNA transfection approach as shown in Figure 4.19 (a-b), however unexpectedly depletion of LRSAM1 did not change the levels of E6-AP mRNA as observed by RT-PCR analysis (Figure 4.19 (a-c)).

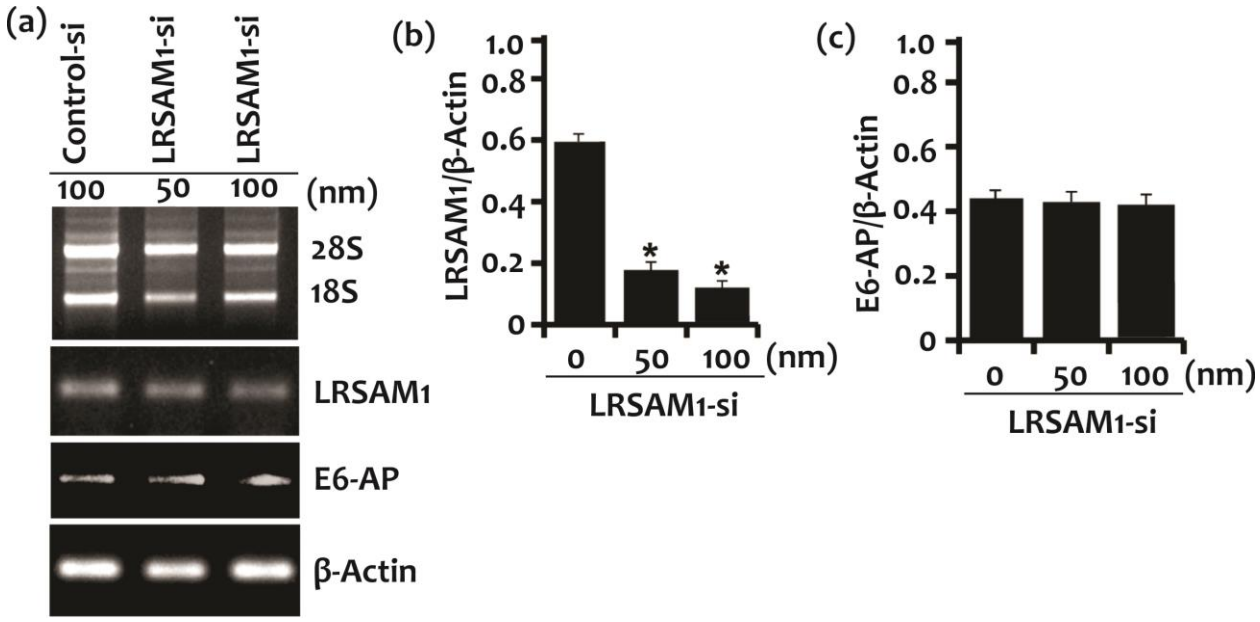

Figure 4.19 : *Analysis of knockdown of LRSAM1 on E6-AP mRNA:* (a) A549 cells transfected either with control-si or LRSAM1-si oligonucleotides were used for RNA collection and RT-PCR using different primers of LRSAM1, E6-AP, and β-actin. (b) The band intensities of LRSAM1/β-actin from (a) is then quantified and presented, where values obtained are mean±SD. (c) Band intensities of E6-AP/β-actin were enumerated and presented, and the values are estimated from mean±SD.

But at the protein level, knockdown of LRSAM1 resulted in E6-AP accumulation in A549 cells (Figure 4.20), and in the same cells we also checked the amount of E6-AP substrates i.e., p53, p27 and p53 transcription-dependent p21 protein. Samples expressing control-si and LRSAM1-si were evaluated by immunoblotting, and the blots were developed with p53, p27, p21, and GAPDH antibodies. As shown in Figure 4.20, the effects of LRSAM1 depletion are thus consistent on E6-AP functional aspects and most likely over accumulated E6-AP decreases p53, p27, and p21 proteins.

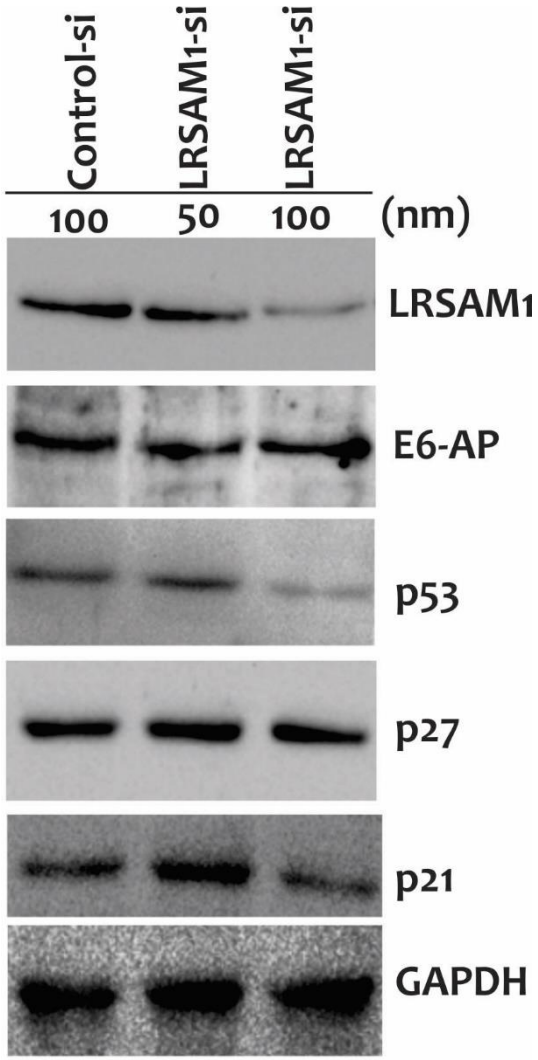

Figure 4.20 : *LRSAM1 knockdown mediated E6-AP accumulation causes cell cycle protein depletion:* LRSAM1 knockdown in A549 cells was performed, and immunoblots of the cells were performed using antibodies of LRSAM1, E6-AP, p53, p27, p21, and GAPDH.

As represented in Figure 4.21, immunofluorescences analysis of LRSAM1, E6-AP, p53, p27 and p21 under depletion of LRSAM1 confirmed the previous results that LRSAM1 depletion upregulates the level of E6-AP and consequently caused a decrease in E6-AP substrates cell cycle proteins, i.e. p53, p27, and p21.

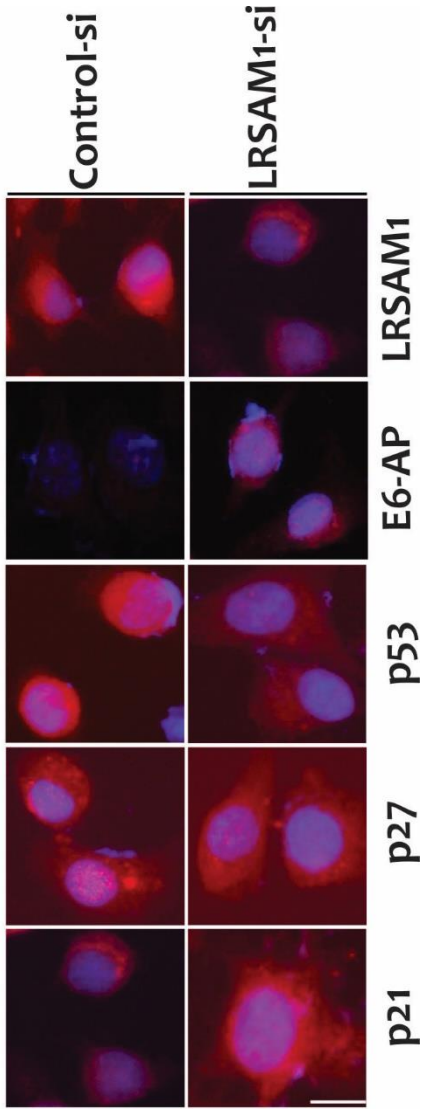

Figure 4.21 : *Immunocytochemistry of LRSAM1 knockdown shows depletion in E6-AP cell cycle substrate proteins:* A549 cells transfected with siRNA oligonucleotides of scramble-si and LRSAM1-si were used for immunocytochemistry by the help of LRSAM1, E6-AP, p53, p27, and p21 antibodies. Nuclei were stained using DAPI. Scale Bar: 20 µm

Most likely LRSAM1 target E6-AP for degradation using proteasomal machinery, which is might be needed in PQC mechanism response against the accumulation of misfolded proteins in different stress generating conditions.

4.2 DISCUSSION

Various complex and proactive processes may engage multiple types of molecular chaperones, E3 ubiquitin ligases, proteasome assembly, and autophagy mechanism form a cellular PQC proteostasis functional network [Amm *et al.*, 2014, Arias and Cuervo, 2011, Bukau *et al.*, 2006]. The chaperones mainly promote protein folding and other components of PQC mechanism direct the misfolded proteins for degradation [Hartl, 1996, Hartl *et al.*, 2011]. Under stress situations, several transcription factors, regulatory proteins, and components of PQC actively contribute to the clearance of disordered or misfolded aberrant proteins [Gidalevitz *et al.*, 2011]. After the elimination of abnormal proteins from the cytosolic pool, how cells further re-establish the survival, stability, and overall response against the normalization of PQC

induced components is poorly understood. Our current finding suggests that LRSAM1 targets E6-AP for degradation using the machinery of the proteasome. For the first time our study indicates how LRSAM1 E3 ubiquitin ligase recognizes endogenous E6-AP for the proteasomal degradation, this systematic action is may be helpful against proteostasis disturbance under cellular stress conditions and may also explain LRSAM1 functions in neurodevelopmental disorder like Angelman syndrome, where E6-AP functions are lost.

Preceding reports have identified that E6-AP is an E3 ubiquitin ligase protein expressed by the gene UBE3A and genetic mutations of the gene leads to maternal imprinting which is the cause of Angelman syndrome, and is often characterized by symptoms like recurrent laughter, loss in gait, ataxia, shivering or tremors, neurological deficits and seizures [Kishino *et al.*, 1997]. Further, the disease is also identified by different bone abnormalities like brachycephaly, microcephaly, osteoporosis and a reduced bone development [Coppola *et al.*, 2007, Kyllerman, 2013, Oiglane-Shlik *et al.*, 2005, Thibert *et al.*, 2013]. Various findings have indicated that E6-AP E3 ubiquitin ligase may modulate different cell cycle regulatory proteins like p53, p27 and thus E6-AP in AS pathogenesis can also contribute by deregulation of cell cycle proteins [Mishra *et al.*, 2009, Mishra and Jana, 2008]. However, it is not well known by which internal molecular mechanism; cells regulate functions of E6-AP protein and in overall modulate the endogenous turnover levels of AS associated E6-AP protein. In our preliminarily routine course of experiments, we have observed clear recruitment of E6-AP and LRSAM1 in the form of aggresomes like inclusions under different critical cellular stress conditions. In immunofluorescence images analysis, we noticed that under heat stress treatment, autophagic dysfunction and proteasomal stress exposures both LRSAM1 as well as E6-AP protein co-localized and accumulated in the nuclear peripheral region.

Earlier reports observed that ER stress, oxidative stress, proteasomal dysfunctions, autophagy inhibition, and heat stress conditions promote the impairment of PQC [Brownstein *et al.*, 2017, Menendez-Benito *et al.*, 2005]. Few critical E3 ubiquitin ligases try their best efforts against such proteotoxic insults and their continuous unprecedented quality control functions provides help in cellular survival and fitness [Fang *et al.*, 2011, Liu *et al.*, 2016]. Next, it was imperative for us to check the functional importance of the subcellular co-localization of E6-AP with LRSAM1 protein. To study the direct effect of LRSAM1 on the E6-AP protein level, we exogenously overexpressed LRSAM1 and notice a reduction of E6-AP in the presence of exogenously expressed LRSAM1 and the same effect was not present in another E3 ubiquitin ligase, i.e. CHIP. It was also observed that mutant or defective (LRSAM1ΔRING) catalytically inactive form was ineffective in the degradation of E6-AP protein. Thus, here we establish the effect of LRSAM1 on E6-AP endogenous levels and the specific effect was confirmed by the use of a mutant or defective form of LRSAM1 under similar conditions. In the next experiments, we further show LRSAM1 induces E6-AP degradation in a concentration-dependent manner. Together, these results enable us to understand the possible novel function of LRSAM1 linked with the degradation of E6-AP.

The earlier finding shows Human papillomavirus type 16 E6 stimulates the degradation of E6-AP [Kao *et al.*, 2000], which give us an idea and raise the possibility of regular and control degradation of E6-AP can contribute in the overall cellular fitness. Interestingly in similar context another report suggests cochaperone/ubiquitin ligase carboxy terminus of Hsp70-binding protein (CHIP) during stress conditions in a sequential manner first ubiquitinates the chaperone-associated misfolded protein and later in the stress recovery phase induces the degradation of Hsp70. The modulation of Hsp70 turn-over during the stress recovery phase

ensures a normal concentration of the chaperone in the cytoplasm and also helps in the maintenance of proteostasis [Qian *et al.*, 2006]. Overall, these studies indicate that LRSAM1 may also possibly retain a novel capability to specifically recognize critical substrates under stress conditions and thereby degrades them after the clearance of misfolded or abnormal proteins. To answer this underlying question of LRSAM1 mediated E6-AP regulation, we further evaluated the LRSAM1 effect on E6-AP endogenous levels. Immunofluorescence analysis results recommend that E6-AP was depleted in the cells overexpressing LRSAM1 and an over-accumulation of E6-AP, and its co-localization was present with mutant LRSAM1ΔRING form. This observation proposes a new possible role of LRSAM1 in cellular protein homeostasis mechanism, which connects a possible link between the maintenance of QC components and its critical substrates.

Above results raised an intriguing possibility to understand how LRSAM1 could contribute to the cellular physiological functions, which can accelerate the clearance of over-accumulated proteins. To test this possibility, we overexpressed LRSAM1 and observe its effects on E6-AP proteasome substrates-dependent cellular physiological mechanisms. Following overexpression of LRSAM1 mediated degradation of E6-AP E3 ubiquitin ligase stimulates the accumulation of E6-AP critical substrates such as p53 [Mishra and Jana, 2008] and p27 [Mishra *et al.*, 2009] which can suppress cellular proliferation. The use of MG132 (a putative proteasome inhibitor) retrogresses the effect of LRSAM1 on E6-AP levels. Several findings have suggested a decisive role of p53 and p27 in both cell cycle regulations and apoptosis [Abbastabar *et al.*, 2018, Kastan *et al.*, 1995, Mercer, 1992]; but how the mechanism by which E3 ubiquitin ligases regulate the cell cycle and apoptosis is still less understood.

Our results suggest that the endogenous levels of E6-AP could be controlled via degradation induced by overexpression of LRSAM1 E3 ubiquitin ligase, however, endogenous LRSAM1 effect on the level of E6-AP needs to be investigated. To answer this notion, we have performed siRNA based knockdown analysis and hence transiently transfected LRSAM1-siRNA and control (scrambled siRNA) in cells. After confirming a knockdown of LRSAM1 and depletion in the endogenous levels of LRSAM1, we demonstrated a marginal endogenous accumulation of E6-AP. Finding an elevation in the E6-AP levels in LRSAM1 partially depleted cells suggest most likely that LRSAM1 targets over elevated E6-AP protein, which was needful for the degradation of the misfolded proteins under stress events. This observation may also help to provide inputs on the role of UPS in cellular stresses and irregular cellular proliferation [Abbas and Dutta, 2017, Daulny and Tansey, 2009, Kelly *et al.*, 2007, Launay *et al.*, 2013].

Previous findings denote that cells always try to gain temporarily tolerance against stress conditions by activation of critical survival pathways, including cellular PQC mechanism [Ma and Hendershot, 2004, Reddy *et al.*, 2018]. But how the normalization of these conserved set of quality control macromolecules is achieved after survival against stress insults is still an emerging question. With the available supportive results and earlier reports, it was essential for us to monitor the overall turnover of E6-AP in the presence of LRSAM1 E3 ubiquitin ligase. Therefore, we have performed a detailed cycloheximide chase experiment of LRSAM1 or control overexpressing cells with or without proteasome inhibitor. In cycloheximide chase experiment, the presence of LRSAM1 induces the turnover of E6-AP protein and further adding MG132 leads to the accumulation of E6-AP, which signifies the revert effect against LRSAM1 function. This observation provides a strong clue that LRSAM1 is maybe involved in the modulation of E6-AP turnover and which can also indirectly contribute in the regulation of other important cellular mechanisms.

In AS mice, deficiency of E6-AP produces a decrease in length of spine dendrites and E6-AP expression in brain areas of hippocampus, cerebellum, cortex layers are needed for maturation of neo-cortex [Dindot *et al.*, 2008, Yashiro *et al.*, 2009]. Other findings of AS mice have suggested a loss in the function of the E6-AP causes a defect in the morphology of polarized dendrite in the pyramidal neurons and a reduction in synaptic vesicles numbers in the hippocampus [Miao *et al.*, 2013, Su *et al.*, 2011]. However, it is still unknown how the loss of functions of E6-AP significantly contributes in the complex molecular pathomechanism of AS disorder. For example, aberrant E6-AP functions can cause an unwanted build-up of its substrate, which may contribute to the pathogenesis of AS disorder. To understand this question, we have applied again the knockdown approach, which can partially deplete the endogenous levels of LRSAM1, and we used these samples for the observation of E6-AP substrates. Here, we have found out a partial depletion of LRSAM1 caused E6-AP protein accumulation, and similar sets of samples retain depletion in the levels of E6-AP substrates p53 and p27, including p53 transcription-dependent p21 protein.

Interestingly a report suggests that a loss in E6-AP functions in AS mice produces an imbalance in the excitatory/inhibitory neurotransmission in the brain and may make AS mice more susceptible to the attack of seizures as is usually observed in the conditions of AS [Wallace *et al.*, 2012]. Similarly in another study, functions of E6-AP to maintain the plasticity of the brain was demonstrated, where an E6-AP deficient AS mice has type5 metabotropic glutamate receptor-dependent depression long term potentiation [Pignatelli *et al.*, 2014]. In summary our finding suggests that most probably LRSAM1 recognizes E6-AP for proteasomal dependent clearance, which can be an essential need of PQC mechanism specifically when cells try to cope up against misfolded proteins mediated stress conditions. This study is also critical for a better understanding of AS disorder and in identifying new possible interplay mechanisms which could modulate E6-AP and may have applications in introducing possible therapeutic interventions in the AS pathology.

4.3 CONCLUDING REMARKS

This study demonstrates the co-localization of E3 ubiquitin ligase LRSAM1 with a QC E3 ubiquitin ligase E6-AP in protein inclusion bodies under different cytotoxic stresses, indicating an association of LRSAM1 with PQC protein E6-AP. Further, LRSAM1 overexpression was found to degrade E6-AP by proteasomal machinery that suggests LRSAM1 may modulate the increased levels of E6-AP, often present in different stresses. Depletion of E6-AP also affects its cell cycle substrate proteins like p53 and p27, and causes their accumulation, which resulted in reduced cellular proliferation. Endogenous LRSAM1 also controls E6-AP, and depletion of LRSAM1 using LRSAM1-si causes E6-AP accumulation with a reduction observed in cell cycle proteins, i.e. p53, p27 and p21. Decrease of cell cycle proteins in LRSAM1-si transfected cells also increased cellular division with time. All these findings suggest LRSAM1 be critical not only in PQC mechanism mediated protein homeostasis in different stresses but also in neurodevelopmental disorder like Angelman syndrome, where E6-AP protein is found to be non-functional.

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