Title page

PML-RARα enhances autophagic activity through inhibiting Akt/mTOR pathway

Running title: PML-RARα and autophagy activation

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Abstract

Autophagy is a highly conserved, closely-regulated homeostatic cellular activity that allows for the bulk degradation of long-lived proteins and cytoplasmic organelles. Its roles in cancer initiation and progression and in determining the response of tumor cells to anticancer therapy are complicated, and the potential significance of autophagy in the pathogenesis and therapeutic response of acute myeloid leukemia has a little investigation. Here, we demonstrate that induction of acute promyelocytic leukemia (APL)-specific PML-RARα but not PLZF-RARα/NPM-RARα fusion protein up-regulates constitutive autophagic activation in leukemic and non-leukemic cells. The significant increase of autophagic activity is also found in the leukemic cells infiltrated bone marrow, spleen and liver from PML-RARα-transgenic APL syngenic transplanted mice. The specific autophagy inhibitor 3-methyladenine significantly abrogates PML-RARα-increased autophagy, while the autophagic flux assay reveals that the fusion protein induces autophagy in the manner of on-rate increase. Furthermore, this autophagy modulation of PML-RARα is possibly mediated by decreased activation of Akt/mTOR pathway. Given the critical role of the PML-RARα oncoprotein in APL pathogenesis, this study would shed new sights for an important role of autophagy in the development and treatment of this disease.

Keywords: autophagy; acute promyelocytic leukemia (APL); PML-RARα; mTOR

Introduction

Autophagy or "self-eating" is a highly conserved, homeostatic process that allows for the bulk degradation of long-lived proteins and cytoplasmic organelles in eukaryotes[1]. During autophagy, portions of cytoplasm and organelles are first engulfed within a double-membrane vesicle called autophagosome, and then the autophagosome fuses with the lysosomes to degrade the sequestered materials by various lysosomal hydrolytic enzymes, followed by generation of amino acids that are recycled for macromolecular synthesis and energy production[2]. The molecular mechanism of autophagy has been widely investigated in yeast and mammalian cells[3,4]. More than 30 autophagy-related genes (*Atg*), such as beclin1, hVps34 (human vacuolar protein sorting factor 34, the homolog of class III phosphatidylinositol 3-kinase (PI3KC3) in yeast), have been identified to participate in autophagic process[2,5-7]. Among these molecules, microtubule-associated protein 1 light chain 3 type I(LC3-I), originally identified as a small subunit of microtubule-associated protein 1A and 1B from rat brain[8], is processed into LC3-II (LC3 conjugating to phosphotidylethanolamine) by a unique protein activation/conjugation system similar to ubiquitylation to form an autophagosomal membrane during autophagy. The conversion of LC3-I into LC3-II is indispensable for both the membrane association of LC3 itself and elongation of the autophagosomal membrane[9, 10].

Autophagy is important in differentiation, development and response to changing environmental stimuli, and plays an important role in numerous diseases, including bacterial and viral infections, neurodegenerative disorders, and cardiovascular disease[2,11]. Recent studies suggest that autophagy may also be essential in the regulation of cancer initiation and progression and in determining the response of tumor cells to anticancer therapy. However, the role of autophagy in these cancer

processes is complicated and may have diametrically opposite consequences for the tumor, as reviewed[12-14]. To our understanding, the potential role of autophagy in the pathogenesis of acute myeloid leukemia (AML), a heterogeneous group of hematopoietic malignancies, has little investigation. Acute promyelocytic leukemia (APL), a unique subtype of AML, is characterized by the blockage of terminal differentiation at promyelocytic stage of hematopoietic stem cells. Almost all cases of APL express oncogenic fusion protein involving in the retinoic acid receptor-alpha (RARα) gene due to the chromosome translocations. More than 95% of APL patients express the PML (for *p*ro*m*yelocytic *l*eukemia)-RARα fusion protein that is formed as a result of the chromosome translocation t(15;17), which is essential for APL pathogenesis[15-18]. More importantly, PML-RARα is directly targeted and degraded by two effective therapeutic agents for APL, *all-trans* retinoic acid (ATRA) and arsenic trioxide (As_2O_3)[19-22]. In addition to PML-RARα, at least five other APL-related RARα-containing fusion proteins, PLZF-RARα, NPM-RARα, NuMA-RARα, STAT5b-RARα, and PRKAR1A-RARα, have been identified and characterized at the molecular level[23, 24]. More recently, it is reported that both ATRA and As_2O_3 induce autophagy via the mammalian target of rapamycin (mTOR) pathway in APL cells and that autophagic degradation significantly contributes to the basal turnover as well as the therapy induced proteolysis of PML-RARα protein[25, 26]. In this work, we provide the first demonstration that expression of PML-RARα but not PLZF-RARα and NPM-RARα fusion proteins can significantly increase constitutive autophagic activity in leukemic and nonleukemic cell lines and *in vivo* APL cells, which is possibly mediated by decreased activation of Akt/mTOR. Given the critical role of the PML-RARα oncoprotein in APL pathogenesis, this study would shed new sights for an important role of autophagy in the development and treatment of this disease.

Materials and Methods

Cell lines and reagents

Human acute leukemic cell line U937 and its subclone U937/PR9 were cultured in RPMI-1640 medium (Sigma-Aldrich, St Louis, MO), supplemented with 10% fetal bovine serum (FBS, HyClone, Logan, UT). HEK293T and U_2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Gaithersburg, MD) with 10% FBS. All cell lines were incubated in a 5% CO_2 / 95% air humidified atmosphere at 37°C. For induction of PML-RARα in U937/PR9 cells, 100μM $ZnSO_4$ was added to the culture medium and PML-RARα expression was confirmed by western blotting. For Induction of autophagy as a positive control, cells were incubated in Earle's balanced salt solution (EBSS), which was made according to the media formulations from Invitrogen Company. 3-methyladenine (3-MA), Pepstatin A and E64d powders were purchased from Sigma-Aldrich (St. Louis, MO) and Calbiochem (San Diego, CA), respectively. 3-MA was dissolved in ultrapure water to form 200mM stock solutions with heating. Pepstatin A and E64 were respectively dissolved in DMSO and ethanol as 10mg/ml stock solutions. Rapamycin obtained from Tocris Bioscience (Ellisville, Missouri) was dissolved in DMSO as 1mM of stock solution.

Plasmids and transfection

Human LC3B cDNA was amplified from leukemic U937 cells by reverse transcription (RT)-PCR and then cloned into pEGFP-C1 expression vector (Clontech) to construct GFP-LC3 plasmid. The sequence of the cDNA insert was confirmed by

sequencing. pSG5-PML-RARα, GFP-PLZF-RARα, and CFP-NPM-RARα plasmids were generous gifts from Dr Shuo Dong in Baylor College of Medicine. DsRed-PML-RARα and HcRed-PLZF-RARα were respectively made by a swap of PML-RARα and PLZF-RARα cDNA into pDsRed-express-C1 and pHcRed-C1 vectors (Clontech) from pSG5-PML-RARα and GFP-PLZF-RARα. Flag-PML-RARα plasmid was generated with the similar strategy into pFlag-CMV4 expression vector (Sigma-Aldrich). Myc-LC3 was obtained as a gift from Dr Qing Zhong in University of California at Berkeley. Transient transfection was performed with Fugene HD transfection reagents according to the manufacturer's procedures (Roche Applied Science, Mannheim, Germany).

Leukemic syngenic transplanted mice and histological analyses

Leukemic blasts from leukemic hMRP8-PML-RARα transgenic mice were collected from BM and spleen by flushing RPMI1640 medium through long bones and dissociated spleens. To generate leukemic mice, $3x10^5$ leukemic blasts were injected into tail vein of 6-8-week-old syngenic FVB/N mice (Shanghai Laboratory of Animal Center, Chinese Academy of Sciences, Shanghai, China) that had been received a sub-lethal irradiation with a total of 3.5Gy. Animal handling was approved by the committee for humane treatment of animals at SJTU-SM. Peripheral blood was obtained from the retro-orbital venous plexus. Bone marrow (BM) cells were extracted by flushing PBS through mouse long bones. Blood and BM smears were prepared according to standard hematological techniques and stained with Wright's Giemsa stain. Spleen and liver specimens were cut into small pieces and fixed in 10% neutral buffered formalin, paraffin embedded and stained with hematoxylin-eosin (H&E).

Transmission electron microscopy (TEM)

Fresh BM cells and the transfected cells were washed in cold PBS, pelleted and subsequently fixed in pre-cooling 2% glutaraldehyde for 2 hours. Cells were then collected by centrifugation, and cell pellets were post-fixed in 1% osmium tetroxide. The samples were then rinsed with phosphate buffer followed by an increasing gradient dehydration step using ethanol and embedded in Epon 812. Ultrathin sections obtained using LEICA Ultracut R were stained with uranyl acetate and lead citrate, and electron micrographs were taken by Philip-CM120 TEM. Data quantification was resulted from total 50 cells analyzed for each sample.

Confocal microscopy and indirect immunofluorescence

U_2OS cells were grown on 13 mm glass coverslips (VWR, West Chester, PA) in 12-well plate and transfected with the indicated plasmids using Fugene HD reagent. Twenty-four hours after transfection, cells were fixed with 4% paraformaldehyde in PBS at room temperature and processed for fluorescence microscopy analysis using confocal microscopy (LEICA TCS SP5). For Myc-LC3 immunostaining process, the fixed cells were stained with rabbit anti-Myc tag antibody (Cell signaling) and then with anti-rabbit IgG-TR (Santa Cruz, CA). Fluorescence signals were observed by LEICA confocal microscopy.

Western blot

The whole cell lysates and tissue lysates were extracted in High Salt Buffer [20mM HEPES (pH 7.9), 20mM NaF, 1mM Na_3VO_4, 1mM $Na_4P_2O_7$, 1mM EDTA, 1mM EGTA, 1mM DTT, 420mM NaCl, 0.5mM PMSF, 20% glycerol, protease and

phosphatase inhibitor cocktails] plus 2×SDS, equally loaded on 8–15% SDS–polyacrylamide gels, and subsequently transferred to nitrocellulose membrane (Amersham Bioscience) and stained with 0.2% Ponceau S red to ensure equal protein loading. After blocking with 5% non-fat milk in PBS for 1 hour at room temperature, the membranes were incubated at 4°C overnight with various specific primary antibodies, and followed by incubation for 1 hour at room temperature with appropriate horseradish peroxidase (HRP)-linked secondary antibodies (Cell Signaling, Beverly, MA). The signals were detected by chemiluminescence phototope-HRP kit (Pierce Biotechnology, Rockford) according to manufacturer's instructions. Polyclonal anti-Beclin1 and anti-Vps34 antibodies were purchased from Novus Biologicals. Antibodies against LC3, RARα, and Atg7 antibodies were purchased respectively from Sigma-Aldrich, Santa Cruz Biotechnology, and ProSci Incorporated. Anti-Grp78 was from Abcam (Cambridge, UK). Other antibodies were purchased from Cell Signaling (Beverly, MA) while p70S6K antibody was obtained from Santa Cruz Biotechnology.

Statistical analysis

Student's t-test was used to evaluate differences between indicated transfections or treatments. A p value of less than 0.05 was considered statistically significant.

Results

Induction of PML-RARα protein triggers constitutive autophagic activation in leukemic and non-leukemic cells

To assess the possible involvement of PML-RARα fusion protein in autophagy, U937/PR9 cell, a subclone of the monocytic leukemia cell line U937 stably transfected with the PML-RARα cDNA under the control of a zinc-inducible promoter[27, 28], was treated with 100μM of Zn_2SO_4 (Zn^{2+}) for different time together with its parental U937 cell line and EBSS incubation as controls. In the presence of Zn^{2+}, PML-RARα protein was induced in U937/PR9 cells (Figure 1A) but not in U937 cells (data not shown). LC3-II was regarded as a faithful marker of autophagy activity, which can be distinguished from LC3-I by SDS-PAGE because of the slightly increased mobility of LC3-II that occurs as a consequence of increased hydrophobicity caused by lipidation[29]. The substitution of growth medium with nutrient-free medium EBSS, a powerful inducer of autophagy[30], increased LC3-II protein in U937/PR9 and U937 cells (Figure 1A). More intriguingly, induction of the PML-RARα protein by Zn^{2+} significantly increased the endogenous LC3-II protein in U937/PR9 cells, which was paralleled with the expression level of this fusion protein (Figure 1A). PML-RARα expression also increased expression of beclin-1 (the mammalian orthologue of yeast Atg6) (Figure 1B), which is another indicator for autophagy initiation and participates in autophagosome formation by interacting with hVps34[31].

As recognized[32], the most reliable and conventional technique to visualize autophagic vacuolization is TEM, because the methods that are currently available for detection of autophagy are affected by numerous intrinsic pitfalls. Considering this, U937/PR9 and U937 were observed under TEM after treated with or without 100μM of Zn^{2+} for 4 hours together with EBSS substitution for 1 hour as the positive control. Autophagic vacuoles (AVs) including double-membrane vesicles or single-membrane vesicles with intracellular contents (cytosol and organelles), namely autophagosomes

and autolysosomes, could be clearly captured in EBSS-treated cells (Figure 1C). Compared with Zn^{2+}-untreated U937/PR9 cells displaying rarely seen AVs with the normal cellular organelles (such as mitochondria), abundant AV-like structures were observed in the cytoplasm of Zn^{2+}-treated U937/PR9 cells (Figure 1C). As summarized in Figure 1D, the percentage of AV^+ cells significantly increased in Zn^{2+}-incubated but not in Zn^{2+}-untreated U937/PR9 cells (left panel, Figure 1D). Among the AV^+ cells, moreover, AV numbers per cell in PML-RARα-expressing U937/PR9 cells were also higher than those in control cells (right panel, Figure 1D). However, Zn^{2+} addition did not produce such an effect in parental U937 cells (Supplemental Fig. S1). All these data supported that PML-RARα expression induces constitutive autophagic activation in leukemic U937 cells.

To address whether the phenomena is cell context-dependent, the PML-RARα fusion gene-expressing plasmid was transfected into human osteosarcoma cell line U_2OS and embryonic kidney cell line HEK293T cells. The results revealed that ectopic expression of PML-RARα also induced autophagy in these cells, as judged by increased LC3-II protein, beclin-1 expression (Supplemental Fig S2) as well as structure and numbers of AVs under TEM (Supplemental Fig S3 for HEK293T cells and data not shown for U_2OS cells). Moreover, monitoring the distribution of fluorescent protein-tagged LC3 fusion protein can be used to visually track autophagic responses under fluorescence microscopy when cytoplasmic diffuse LC3-I is converted into the punctate LC3-II, which is targeted directly to the preautophagosomal and autophagosomal membranes[29]. Thus, GFP tagged human LC3 plasmid together with DsRed or DsRed-PML-RARα expression vector were transiently transfected into U_2OS cells. After transfection for 24 hours, cells transfected with GFP-LC3 alone were subsequently incubated with EBSS for 1 hour

or treated with 0.5μM of rapamycin (another widely-used autophagy inducer[32]) for 6 hours as positive controls. GFP-LC3$^+$ cells incubated with EBSS or rapamycin showed dramatic transition from the diffuse cytoplasmic pattern to the punctate membrane pattern as assessed by calculating the percentages of punctate GFP-LC3$^+$ cells (10±0.2% for control; 52±0.7% for EBSS; 60.2±3.1% for rapamycin). Compared with DsRed empty vector-transfected cells (13.7±3.1%), intriguingly, there was a significantly higher percentage of GFP-LC3-aggregated foci cells (40.7±2.4%) in DsRed-PML-RARα-transfected cells, which presented a PML-RARα expression-specific microspeckled localization in nucleus due to disruption of PML-nuclear body[23] (Figure 1E). All these results suggested that overexpression of PML-RARα protein induces constitutive autophagic activation in cell type-independent manner.

The constitutive autophagic activity also exists in *in vivo* leukemic cells from PML-RARα-produced leukemic mice

To evaluate the *in vivo* effect of PML-RARα on autophagy, leukemic cells from hMRP8-PML-RARα transgenic mice[33], were intravenously injected into syngenic FVB/N mice. Based on our previous experience[34, 35], we could successfully generate transplant leukemic mice at about 29 days after injection of 3×10^5 cells per mouse, as evidenced by strictly monomorphic and immature promyelocyte-like cells accumulated in peripheral blood, BM as well as liver and spleen (Figure 2A). Then, we compared LC3 protein level in leukemic cell-infiltrated organs between normal and leukemic mice. The results revealed that LC3-II protein significantly increased in BM, spleen, and liver tissues from leukemic mice with PML-RARα expression,

compared to those from normal mice (Figure 2B). Furthermore, TEM observation demonstrated that large numbers of AVs were accumulated in the cytosol of the immature promyelocytes from BM in leukemic mice, compared with cells from BM in normal mice (Figure 2C-D). These results indicate that the constitutive autophagic activity is also presented in *in vivo* leukemic cells from PML-RARα-produced leukemic mice.

Increased autophagic activity can not be seen in APL-specific PLZF-RARα and NPM-RARα fusion proteins

Other rare variant translocations occurred in the APL cases generate fusion proteins disrupting the RARα locus on chromosome 17, such as PLZF-RARα and NPM-RARα[23,36]. To test whether these variant fusion proteins had autophagy-modulating capabilities, U_2OS cells were transiently co-transfected with GFP-LC3 and HcRed-PLZF-RARα or DsRed-PML-RARα expression plasmids, or their corresponding empty vectors. To exclude the disturbance of CFP (CFP-NPM-RARα) and GFP (GFP-LC3) channel overlapping, we used Myc-LC3 plasmid to co-transfected with CFP-NPM-RARα or CFP vectors into U_2OS cells. Different from the PML-RARα-induced GFP-$LC3^+$ punctate structure, HcRed-PLZF-RARα or CFP-NPM-RARα expression did not significantly alter GFP-LC3 or Myc-LC3 localization from the diffuse pattern into the punctate pattern (3A/B). Similarly, overexpression of CFP-NPM-RARα or GFP-PLZF-RARα protein did not increase LC3-II protein (Figure 3C).

Lysosome inhibitors increase while hVps34 inhibitor prevents PML-RARα-

induced constitutive autophagy activation

Although membrane-conjugating LC3-II localized in autophagosome was used to estimate the abundance of autophagosomes before they are degraded through fusion with lysosomes, LC3-II itself is also degraded by lysosomal proteases in the late phase of autophagy[29]. Therefore, the accumulation of autophagosomes induced by PML-RARα could involve an enhanced autophagic sequestration (on-rate increase) or a reduced degradation of autophagic material (off-rate decrease). To distinguish these, we performed "autophagic flux" assay and assessed PML-RARα-induced vacuolization by monitoring the localization of GFP-LC3 and LC3 conversion, either in the presence or absence of the cysteine protease inhibitor E64d and the aspartic protease inhibitor Pepstatin A. Consistent with the previous reports[29, 32], addition of these two protease inhibitors to the starvation medium could effectively inhibit the degradation of lysosomal protein, resulting in further accumulation of LC3-II and the increase of GFP-LC3 puncta (Supplemental Fig. S4A/B). Intriguingly, the inhibitors also increased PML-RARα-triggered accumulation of GFP-LC3 puncta (Figure 4A) and LC3-II protein (Figure 4B), suggesting that PML-RARα enhances autophagic activity in the manner of on-rate increase of AV formation.

Furthermore, distinct classes of PI3Ks are involved in signaling pathways that control autophagy in mammalian cells. Initiation of the autophagy process requires hVps34 and its complex formation with beclin1 and protein kinase 150[37]. Addition of 3-MA, which prevents autophagy at the sequestration step[38], could significantly reduce EBSS-induced GFP-LC3$^+$ cells with punctate foci and LC3 conversion (Supplemental Fig S4A/C). Thus, U_2OS cells were transiently transfected with DsRed-PML-RARα or DsRed expression vectors for 24 hours, followed by treatment

with or without 10 mM of 3-MA for additional 4 hours. As shown in Figure 4A, 3-MA treatment significantly suppressed the increase of punctate GFP-LC3$^+$ cells induced by PML-RARα. Similarly, 3-MA also eliminated LC3-II protein accumulation induced by PML-RARα expression (Figure 4C). Of note, our results showed that the constitutive autophagy activation induced by the fusion protein did not accelerate the degradation of ectopic expression of PML-RARα (Figure 4C).

PML-RARα-induced autophagic activation possibly mediated by decreased Akt/ mTOR activation in PR9 cells

To ask whether the PML-RARα-induced constitutive autophagy activation involves in Akt-mTOR signaling pathway, we examined the phosphorylation state of Akt and the downstream substrates of mTOR. The results showed that the Akt activation, as determined by its phosphorylation at Thr308 and Ser473, was significantly inhibited in PML-RARα-expressing cells. After induction of PML-RARα protein in Zn$^+$-treated U937/PR9 cells, mTOR protein level had no change but its activation was significantly reduced in U937/PR9 cells, as assessed by the decreased phosphorylation of p70-S6K1 and 4E-BP1(Figure 5A), two direct substrates of mTOR[39]. However, these alterations did not occur in Zn$^+$-treated and -untreated U937 cells (Figure 5B).

Discussion

Autophagy, which occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover, is rapidly upregulated

when cells need to generate intracellular nutrients and energy during starvation, growth factor withdrawal, or high bioenergetic demands. Such upregulation of autophagy also occurs when cells are preparing to undergo structural remodeling such as during developmental transitions or to rid themselves of damaging cytoplasmic components during some poor environment such as oxidative stress, infection, or protein aggregate accumulation[40]. The regulation of autophagy overlaps closely with signaling pathways that regulate tumorigenesis[12-14, 40]. In the present study, we demonstrated that induction of PML-RARα protein could significantly upregulate constitutive autophagy activation in leukemic U937 cells, as evidenced by the increase of LC3-positive punctate structures, elevation of LC3-II and beclin1 protein levels, and cytoplasmic AV-like structure accumulation under TEM. This phenomenon could also be seen in other two non-leukemic cells with transient transfection of PML-RARα expressing plasmids. These results proposed that the upregulating effect of the PML-RARα fusion protein on autophagy was independent of the cellular context. Besides PML-RARα fusion protein, the rare APL cases also express other fusion proteins due to different chromosome translocations which always involve RARα gene in chromosome 15. *In vitro* and transgenic mice analysis showed that all these fusion proteins are critical of the pathogenesis of APL[23, 41]. Therefore, we also investigated the potential effect of other two APL-related fusion proteins, PLZF-RARα and NPM-RARα, on the autophagy. Unlike PML-RARα, unexpectedly, these two fusion proteins has no significant effect on constitutive autophagy formation, as estimated by multiple autophagy-assessing assays[32]. To answer whether the enhanced effect of PML-RARα overexpression on autophagy activation is artificial in *in vitro* transfected effect, we further investigated leukemic cells derived from PML-RARα-transgene-initiating APL-like mice. Compared with cells from wild

mice, the APL-like cells infiltrated into BM, spleen and liver from the transplanted mice also presented increased constitutive autophagy activation. All these data supported that PML-RARα enhances autophagy generation.

Autophagy involves the delivery of cytoplasmic cargo sequestered inside double-membrane vesicles to the lysosome, where the captured material, together with the inner membrane, is degraded[40]. Our results demonstrated that lysosomal protease inhibitors E64d and Pepstatin A increased PML-RARα-triggered autophagy activation, indicating that the upregulated autophagy of PML-RARα is not due to the alterations of autolysosomal event. On the other hand, 3-MA preventing autophagy at the sequestration step could significantly reduce the increase of autophagy induced by PML-RARα, indicating that PML-RARα induces autophagy in the manner of on-rate increase of autophagosome formation.

The observation that PML-RARα expression favors mammalian cells to undergo autophagy led to the consideration that the alteration of autophagy-related signaling molecules possibly contributed to PML-RARα-enhanced autophagic activity. It has found that autophagy is regulated by a series of signaling molecules such as Atg7 and Vps34, as widely reviewed[5, 6]. Our results showed there was no significant difference of Atg7 and Vps34 expressions in the presence and absence of PML-RARα induction (data not shown). More recently, it has been demonstrated that endoplasmic reticulum stress-related autophagic modulator Grp78 was up-regulated in more than 10% AML patients with a favorable prognosis and Grp78va, a variant form of Grp78, was found to increase in several human leukemic cells and leukemia patients[42, 43]. However, Grp78 expression was also not significantly altered by transient transfection of PML-RARα plasmid in U_2OS and 293T cells (data not shown). Considering that the promotion of autophagic activity was only specifically produced by PML-RARα but

not PLZF-RARα and NPM-RARα expression, we extrapolated that PML-RARα-induced autophagic activity involves its PML motif. Previous studies showed that PML interacts with mTOR, and inhibits its activity by negatively regulating its association with Rheb and further sequestrating mTOR in nucleus under normal and especially in hypoxic conditions[44]. Moreover, PML has also been found to interact with Akt phosphatase PP2a and then dephosphorylate Akt, which further inhibits mTOR activity[45]. mTOR belongs to the phosphatidylinositol kinase-related kinase (PIKK) family, and binds several proteins to form two distinct protein complexes, mTORC1 (mTOR complex 1) and mTORC2. Emerging evidence showed that the inhibition of mTORC1 activity, targeted and regulated by several pathways such as nutrient starvation and reduced growth factor, is a crucial step for autophagy induction in eukaryotes[46]. The growth factor signaling that regulates mTORC1 mainly involves the insulin/insulin-like growth factor-PI3K-Akt pathway, which negatively regulates autophagy induction. Indeed, our data showed that PML-RARα induction also reduced activation of Akt with the decreased phosphorylation of two down-stream substrates of mTORC1. Although it remains to be further explored how PML-RARα inhibits Akt/mTOR activation, our results supported that PML-RARα induced constitutive autophagy activation possibly involved Akt-mTOR1 signaling pathway, resulting in significantly reduced activation of Akt and mTOR pathway, and therefore induction of autophagy.

All of RARα-involving fusion proteins found so far can trigger leukemia with similar or distinct phenotypes in the transgenic mice[23, 47]. However, they present different response to ATRA and As_2O_3 in mice models and human APL patients[47]. For example, PML-RARα but not PLZF-RARα-carrying leukemia is responsive to ATRA or As_2O_3 treatment, which induce protolysis of PML-RARα but not PLZF-RARα

protein. PML-RARα destruction is regarded as essential for induction of terminal differentiation and/or eradication of leukemia-initiating cells of APL[18]. Recently, Isakson et al[25] reported that both ATRA- and As_2O_3-induced autophagy contributes significantly both to the basal turnover as well as the therapy induced proteolysis of PML-RARα in APL cells. They also showed a correlation between autophagy and therapy-induced differentiation of APL cells. By the way, it was also explored that autophagy is a critical mechanism for As_2O_3-induced antileukemic effects[48]. Although we could not find that the increased autophagy was accompanied with acceleration of the degradation of the fusion protein, our results proposed that increased constitutive autophagy activation possibly contributes to effective response of PML-RARα APL patients to ATRA and/or As_2O_3.

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Figure legends

Figure 1. The effect of ectopic expression of PML-RARα on autophagic activity in leukemic and nonleukemic cells. (A-B) U937/PR9 and U937 cells were respectively treated with and without 100 μM $ZnSO_4$ or EBSS for the indicated hours. Cell lysates were harvested for immunoblotting proteins as indicated. (C) TEM micrographs of the indicated treatments in U937/PR9 cells. AV-like structures were pointed by arrowheads. "Nuc" stands for nucleus. The indicated scales on images of control, EBSS and Zn^{2+} treated cells were 1000 nm, and high-magnification picture (right panel of bottom row) was from the framed area in the Zn^{2+}-treated cell (bar: 500 nm). (D) Quantification of data in panel C was calculated and summarized. The percentage of AV^+ cells was shown in the left panel and among these AV^+ cells, the percent cells with indicated AV numbers per AV^+ cell were indicated in the right panel. The symbol * indicates a *p* value of less than 0.05 against U937/PR9 control cells. (E) U_2OS cells were transfected with GFP-LC3 alone (top row) or co-transfected with GFP-LC3 and DsRed-PML-RARα or DsRed vector (bottom row). After 24 hours, cells were examined by confocal microscopy. The representative images of the indicated transfected cells with the corresponding treatment were shown, where values (x±s.d.) represent the percentage of GFP-LC3-puncta positive cells by observing a total of 200 cells for each indicated treatment. For DsRed-PML-RARα-transfected cells, two fields were shown. The symbols * and # respectively indicated *p* values of less than 0.001 and 0.01 compared with the untreated cells with GFP-LC3 transfection alone. The symbol $^{\&}$ indicated a *p* value of less than 0.05 compared with the cells co-transfected with DsRed and GFP-LC3 plasmids.

Figure 2. *In vivo* effect of PML-RARα expression on autophagy in leukemic mice. Leukemic cells (3×10^5) from BM and spleen of APL transgenic mice were injected into FVB/N mice via tail vein. Animals were sacrificed about twenty-nine days after leukemic cell transplantation. (A) Cytologic analysis by Wright's Giemsa staining of peripheral blood (PB) and BM. Histopathological sections of spleen and liver from the indicated mice were stained with H&E. Images were observed under microscope with Nikon digital camera. (B) The proteins from BM cells and tissues of the normal and diseased mice were extracted and the indicated proteins were detected by western blot. (C) Representative electron micrographs of myelocyte and promyelocyte in BM respective from normal and disease mice were observed under TEM. The indicated scales were 2000 nm on the top two images, and the high-magnification picture (bottom panel) was from the white framed area at middle panel (bar: 1000 nm). (D) Percent cells with indicated AV numbers per immature granulocyte in BM from the indicated mice were calculated and summarized.

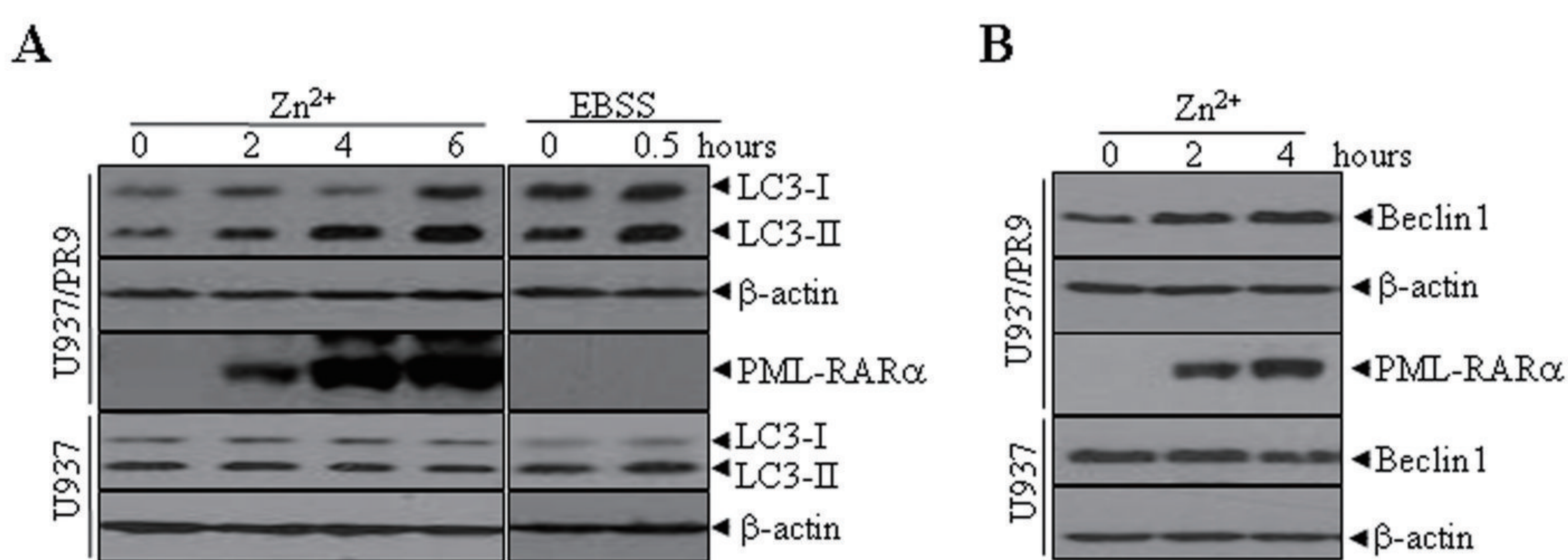
Figure 3. Effects of PLZF-RARα and NPM-RARα fusion proteins on autophagy. U_2OS cells were co-transfected with GFP-LC3 and HcRed-PLZF-RARα or DsRed-PML-RARα plasmids, or corresponding empty vectors (HcRed or DsRed), or were co-transfected with Myc-LC3 and CFP-NPM-RARα or CFP vectors. 300 ng of plasmids were given for each vector. After transfection for 24 hours, the cells were stained with anti-Myc antibody or directly analyzed by confocal microscopy. Myc-LC3 signal was imaged on red fluorescence protein (RFP) channel and CFP signal was obtained on CFP channel. (A) The representative images of cells transfected with the indicated constructs were shown. The arrowheads pointed to cells

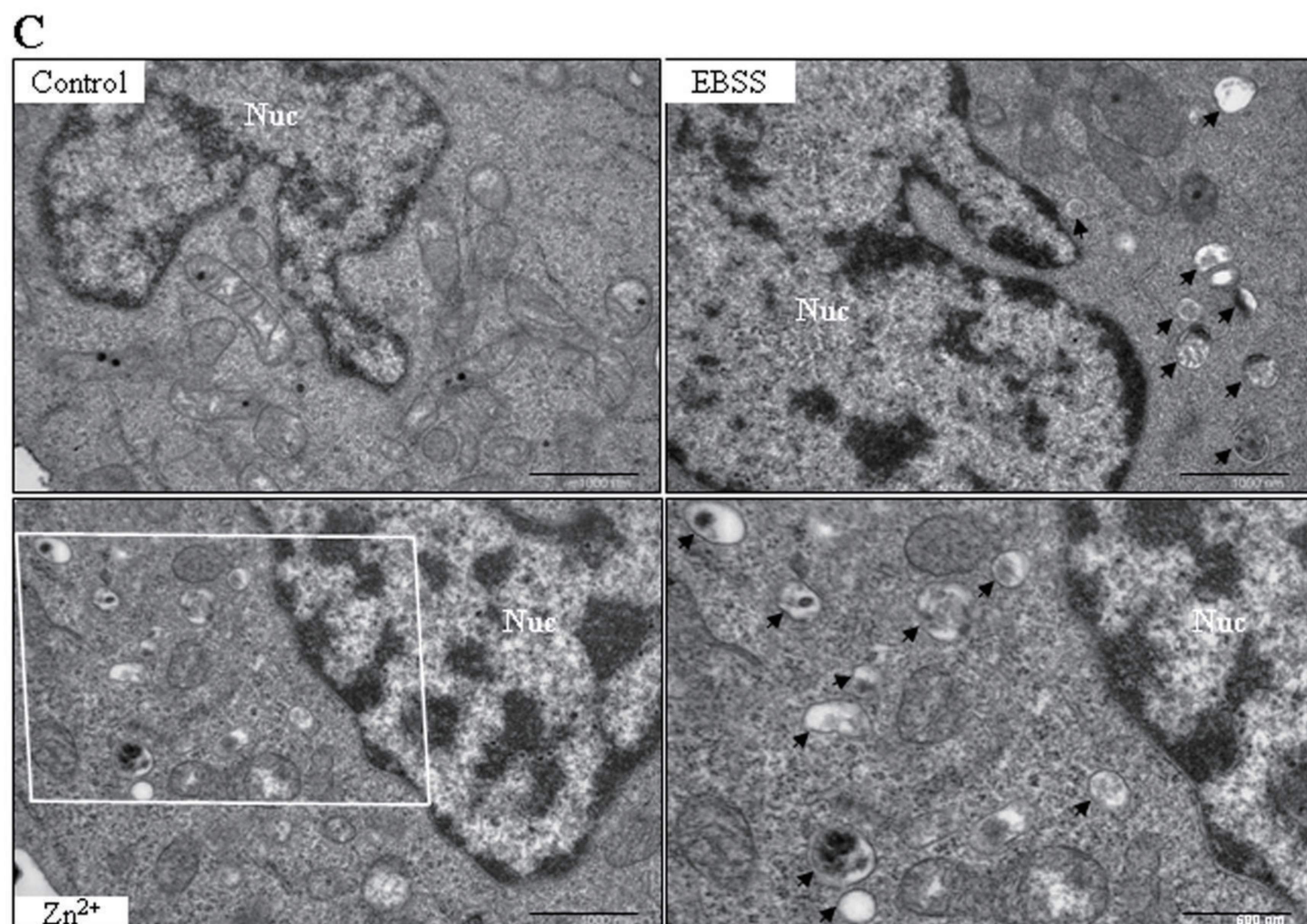
with the expression of proteins as indicated. (B) GFP-LC3$^+$-aggregated cell percentages were calculated and summarized from a total of 200 cells with positive transfection. The symbol * indicates a *p* value of less than 0.001 compared with the cells co-transfected with DsRed and GFP-LC3 plasmids. (C) After transiently transfected with the indicated plasmids, U_2OS cells were extracted and detected by western blot. The transfected expressions of APL-related fusion proteins were confirmed by RARα antibody.

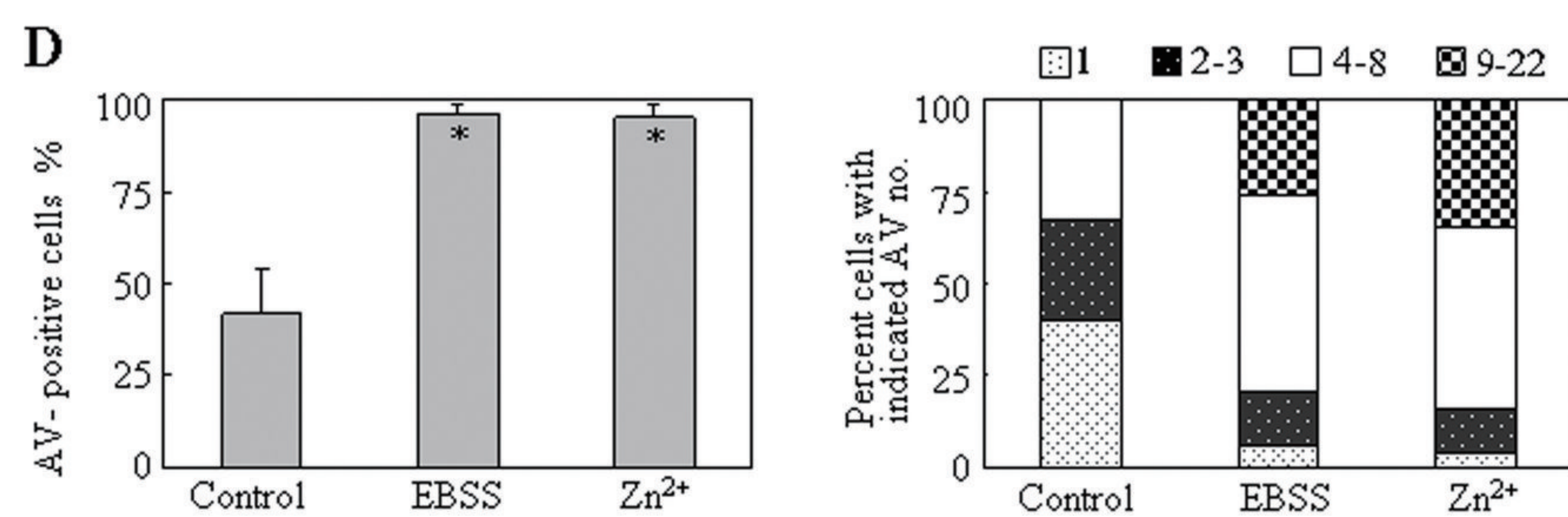
Figure 4. Effects of lysosomal enzyme inhibitors and 3-MA on altered localization and expression pattern of LC3 protein induced by PML-RARα. (A) U_2OS were transiently co-transfected with GFP-LC3 and DsRed-PML-RARα (bottom panels) or DsRed (top panels) for 24 hours, followed by treatment with or without 3-MA (10 mM) and Pepstatin A (10 μg/ml) plus E64d (10 μg/ml) for additional 4 hours. Then, the cells were observed under confocal microscopy. The representative images for each treatment were shown. GFP-LC3-aggregated positive cells % were calculated and summarized from a total of 200 transfected cells for each indicated treatment. Symbol * stands for $p< 0.05$. (B-C) U_2OS were transiently transfected with the indicated concentrations of Flag-PML-RARα expression vector or Flag empty (1.0μg for the transfection concentrations in B) for 24 hours, and then treated with or without Pepstatin A (10 μg/ml) and E64d (10 μg/ml) (B), 3-MA (10 mM) (C) or equal volume of vehicle for additional 4 hours. Cell lysates were harvested and analyzed with western blot by specific antibodies.

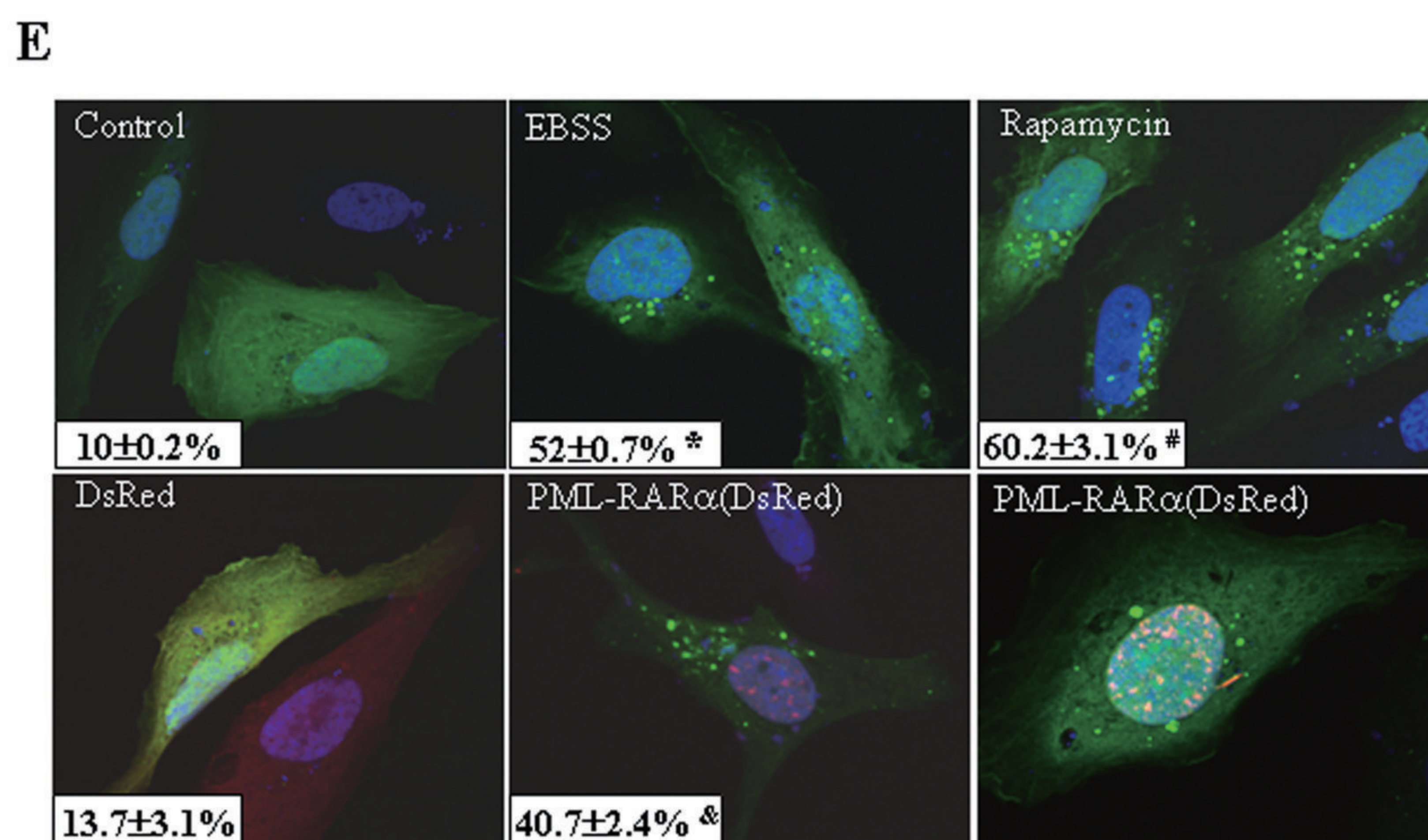
Figure 5 . Effects of PML-RARα overexpression on Akt-mTOR pathway in

leukemic cells. U937/PR9 and U937 cells were treated with or without 100 μM $ZnSO_4$ for 4 hours. The total proteins were harvested and the indicated proteins were analyzed by western blot. The induction of PML-RARα expression in U937/PR9 cells was confirmed by RARα antibody.

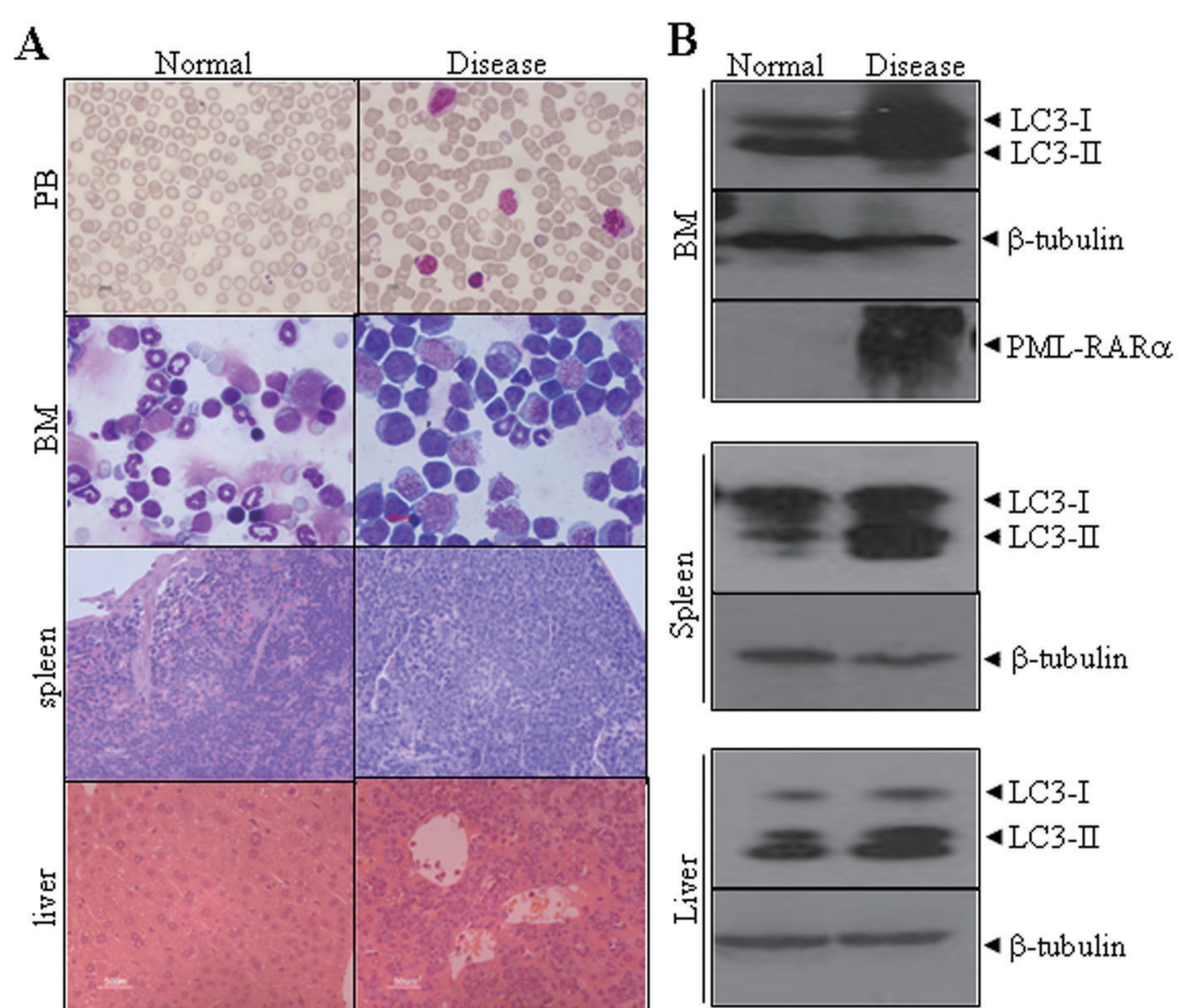


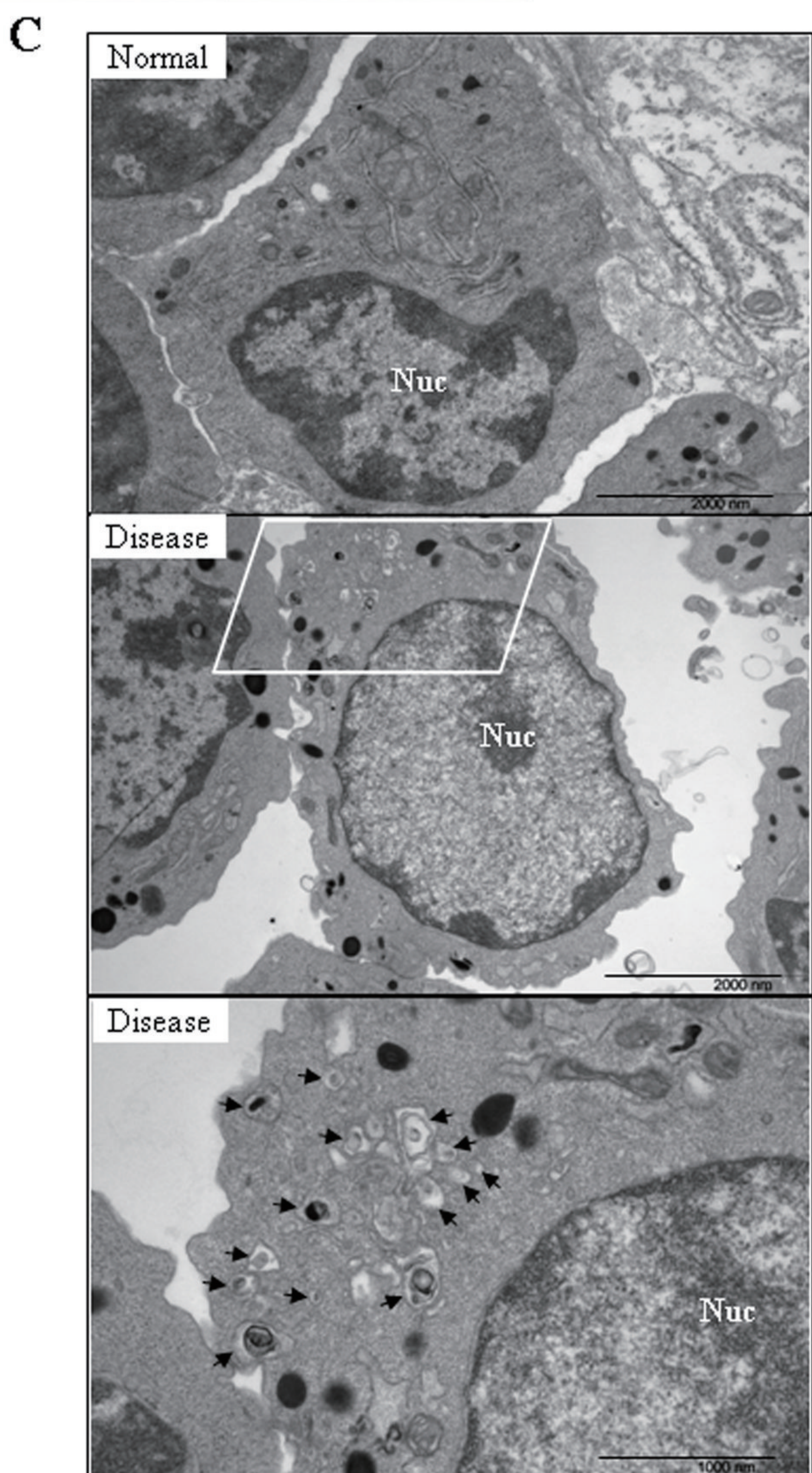


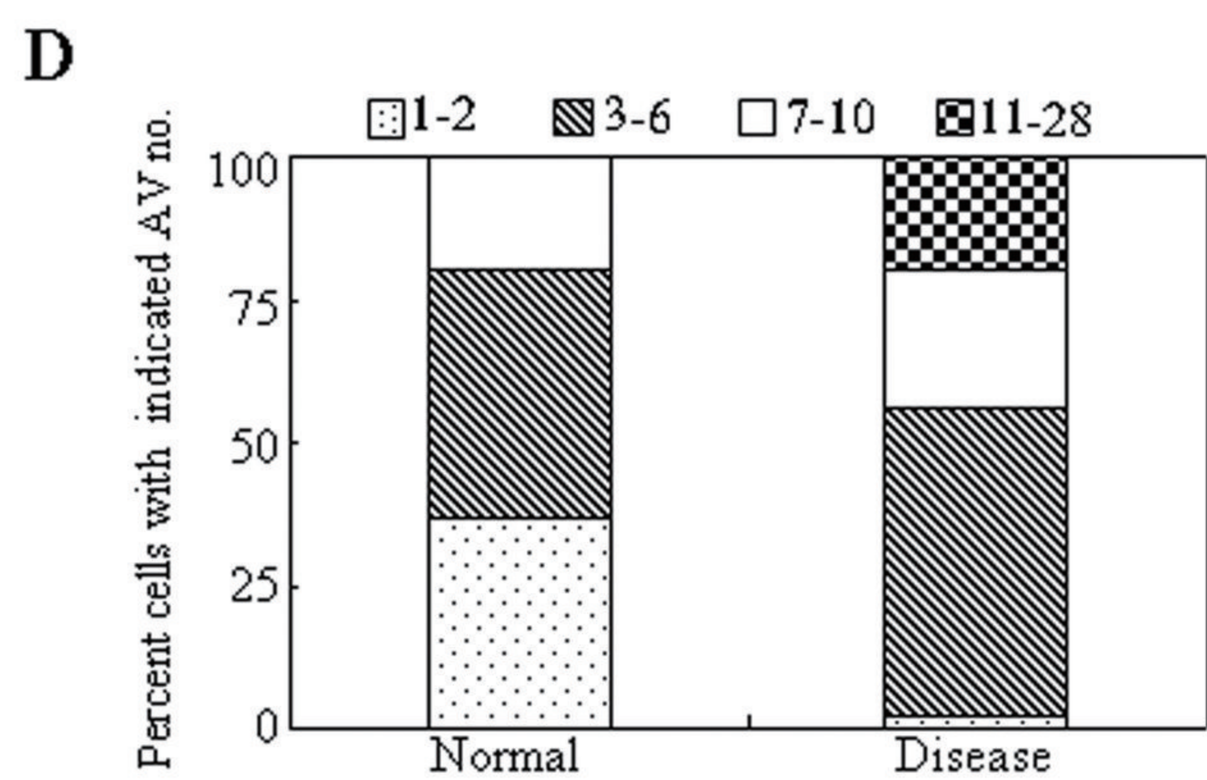




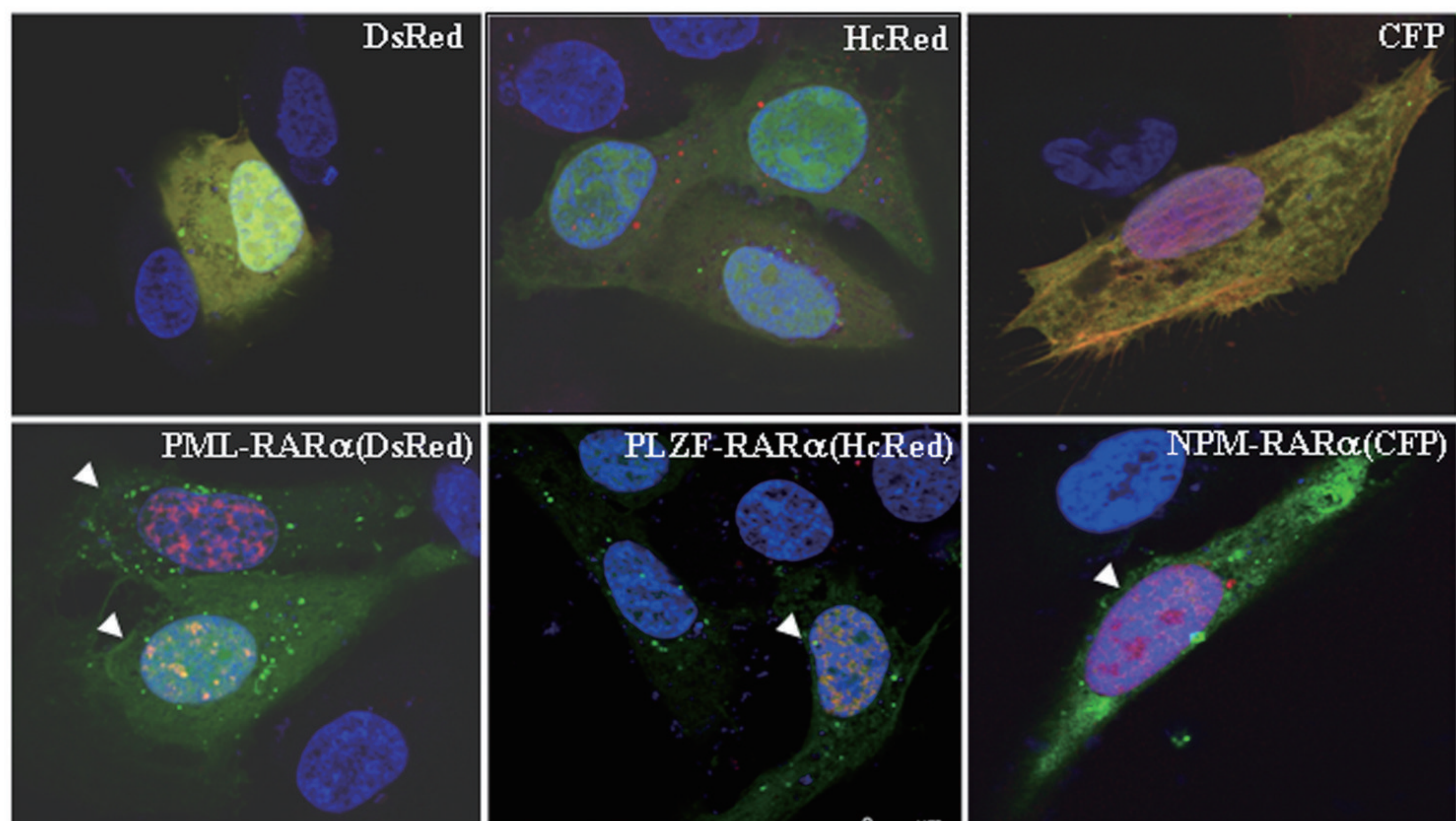
Huang Y et al, Figure 1

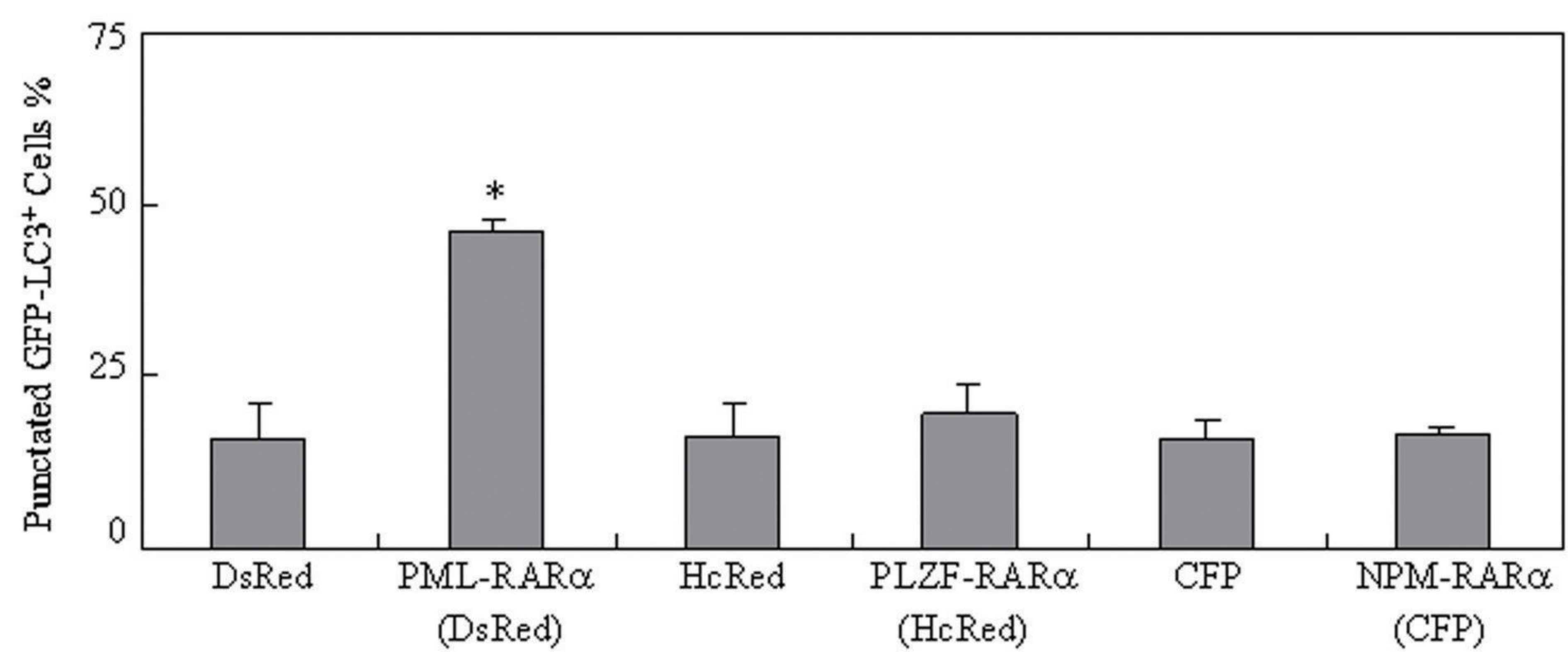


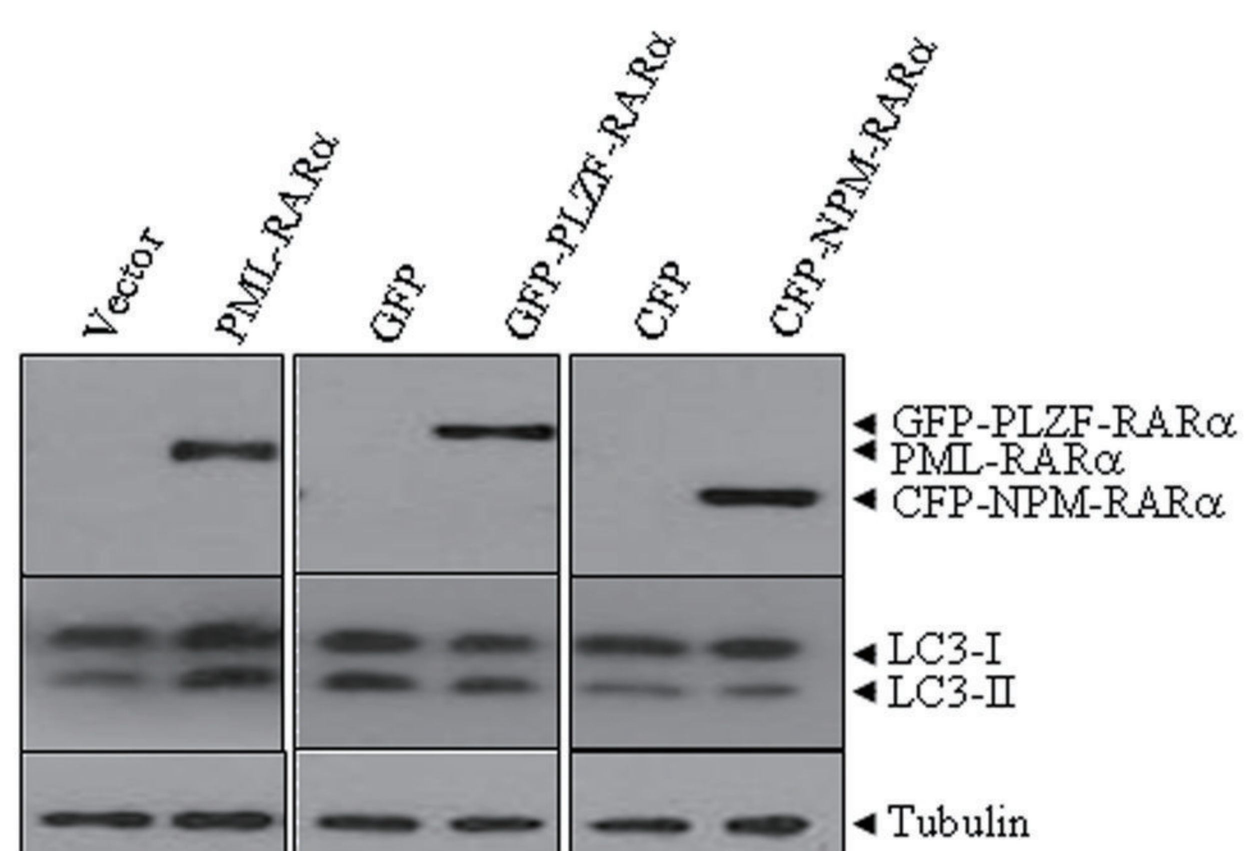




Huang Y et al, Figure 2

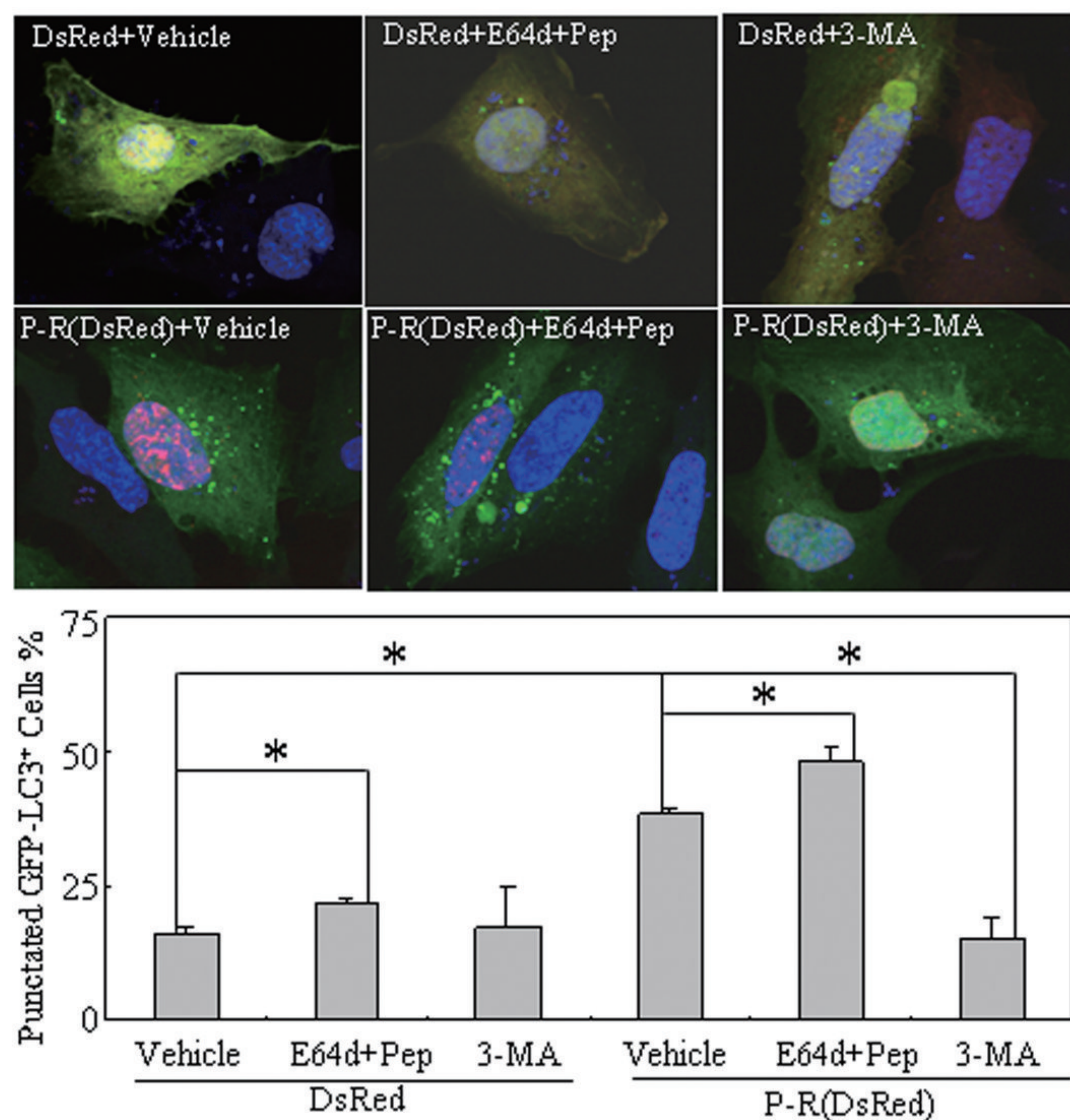






Huang Y et al, Figure 3

A



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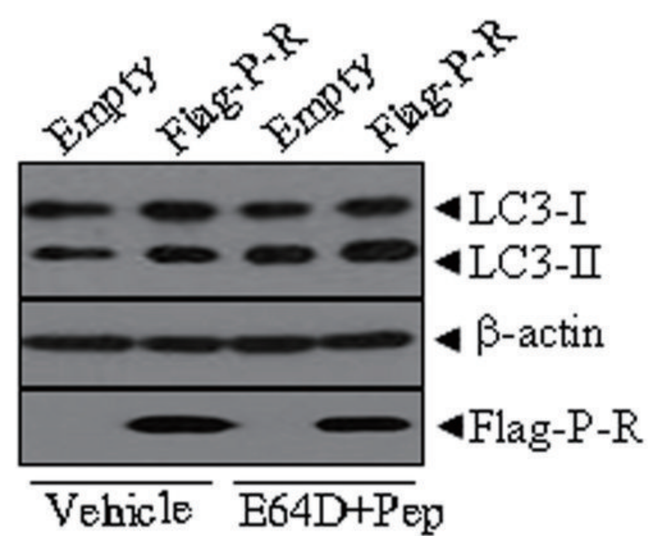

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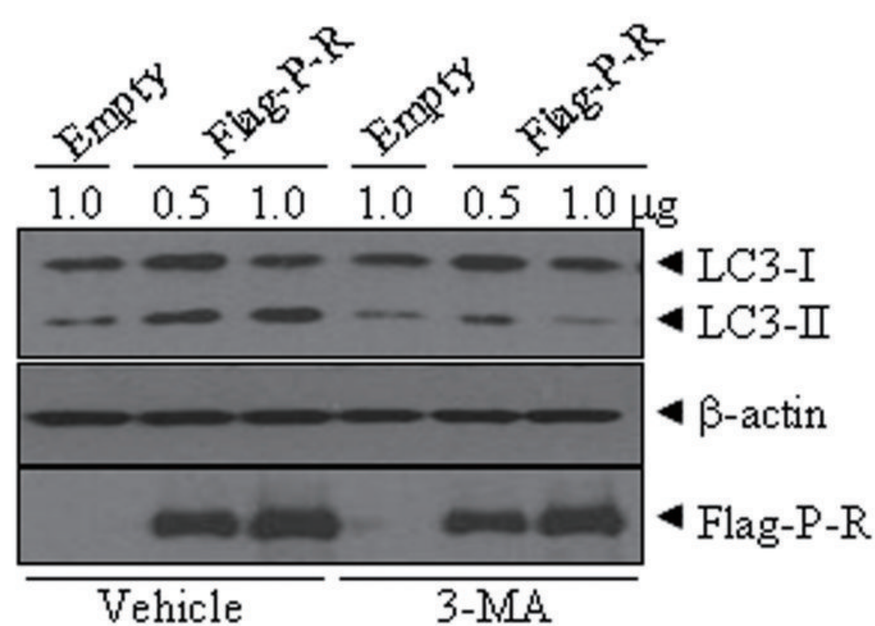
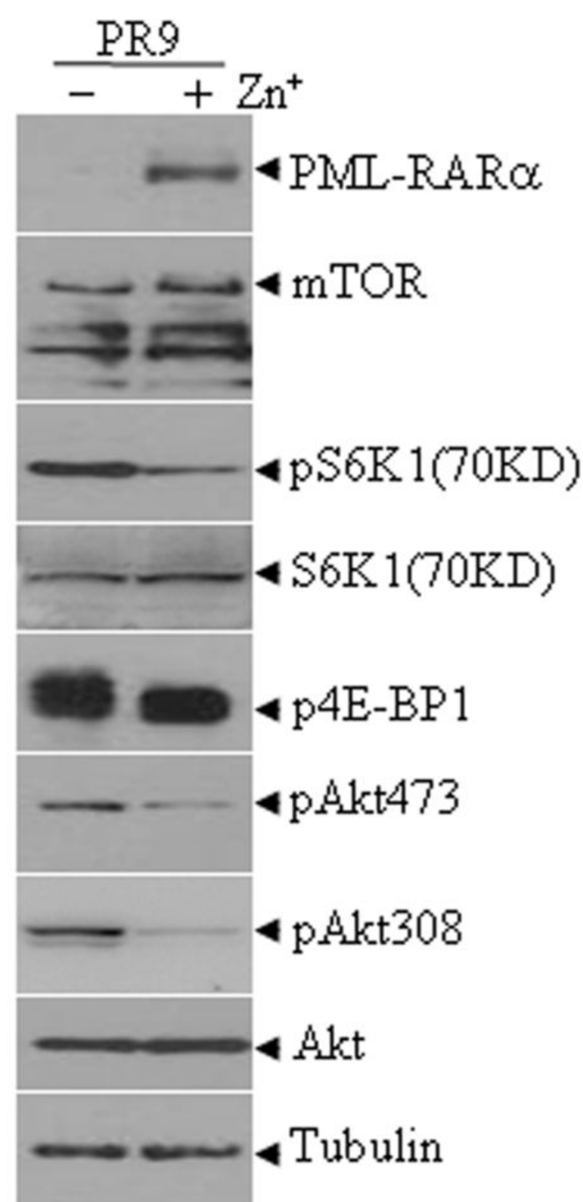


Huang Y et al, Figure 4

A



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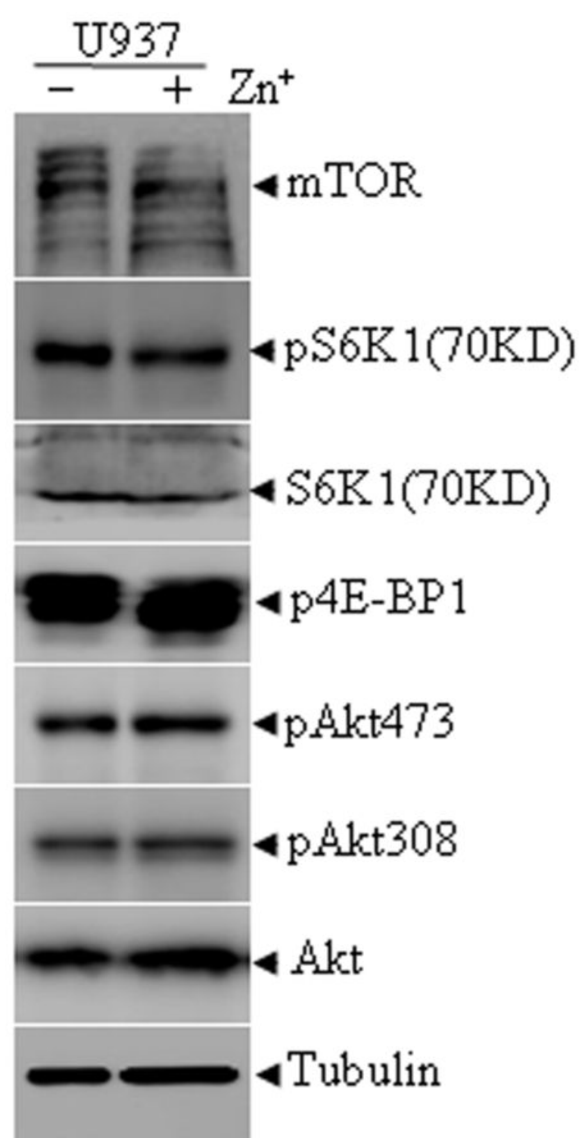

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