

Jun Proteins Are Starvation-Regulated Inhibitors of Autophagy

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Abstract

The growing number of biological functions affected by autophagy ascribes a special significance to identification of factors regulating it. The activator protein-1 (AP-1) transcription factors are involved in most aspects of cellular proliferation, death, or survival, yet no information regarding their involvement in autophagy is available. Here, we show that the AP-1 proteins JunB and c-Jun, but not JunD, c-Fos, or Fra-1, inhibit autophagy. JunB inhibits autophagy induced by starvation, overexpression of a short form of ARF (smARF), a potent inducer of autophagy, or even after rapamycin treatment. In agreement, acute repression of JunB expression, by JunB knockdown, potently induces autophagy. As expected from autophagy-inhibiting proteins, Jun B and c-Jun expression is reduced by starvation. Decrease in JunB mRNA expression and post-transcriptional events downregulate JunB protein expression after starvation. The inhibition of autophagy by JunB is not mediated by mammalian target of rapamycin (mTOR) regulation, as it occurs also in the absence of mTOR activity, and autophagy induced by JunB knockdown is not correlated with changes in mTOR activity. Nevertheless, the transcriptional activities of c-Jun and JunB are required for autophagy inhibition, and JunB incapable of heterodimerizing is a less effective inhibitor of autophagy. Most importantly, inhibition of autophagy in starved HeLa cells by JunB enhances apoptotic cell death. We suggest that JunB and c-Jun are regulators of autophagy whose expression responds to autophagy-inducing signals. *Cancer Res*; 70(6); 2318–27.

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Introduction

Autophagy is a mechanism essential for several physiologic processes including development, differentiation, tissue remodeling, cellular homeostasis, cell survival or death, innate immunity, and pathogenesis (1–5). The wide spectrum of biological activities modulated by autophagy has renewed great interest in the process over the past few years. One of the particularly important subjects is the role of autophagy in the regulation of tumor development, progression, and response to chemotherapy. Interestingly, autophagy emerges as a tumor suppression mechanism (6), a conclusion derived from observations of scenarios in which autophagy is compromised. For example, expression of autophagy-related gene 6/beclin 1 is often decreased in breast, ovarian, and brain tumors (7, 8), and heterozygous disruption of beclin 1 in mice results in increased tumorigenesis, thus underscoring its

haploinsufficient tumor suppressor activity (9, 10). Two mechanisms are currently suggested to account for the ability of autophagy to suppress tumorigenesis (11). First, autophagy prevents genomic instability in metabolically stressed cells (12). Second, in the absence of autophagy, inflammation resulting from enhanced necrosis may stimulate tumorigenesis. Paradoxically, autophagy may also promote the survival of starved tumor cells in tumor parts suffering from poor blood supply. In addition, autophagy may have cytoprotective roles during anticancer therapy (13).

One of the normal physiologic roles of autophagy is the response to metabolic stress induced by nutrient-poor conditions (14). Identification of the molecules regulating autophagy upon starvation has focused on the mammalian target of rapamycin (mTOR), a potent inhibitor of autophagy (15). Several other kinases positively or negatively regulate autophagy. Akt inhibits autophagy and accelerates tumor growth (11), whereas the c-Jun NH₂ terminal protein kinase (JNK), a known regulator of apoptosis, induces autophagy. In this case, JNK1 phosphorylates Bcl2 during starvation, dissociating it from beclin 1, thereby unleashing the latter to engage in autophagy (16).

Several transcription factors were also found to regulate autophagy. These include oncogenes as well as tumor suppressors, including p53 and its upstream regulator p14/p19ARF (13). p53 may enhance autophagy when activated by genotoxic stress through the upregulation of one of its target genes, DRAM (damage-regulated autophagy modulator; refs. 17, 18). Surprisingly, reduced activity of p53 by chemical

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inhibition, knockdown, or knockout also results in the induction of autophagy, suggesting that p53 plays a dual role in regulation of autophagy (19). The tumor suppressor p19/p14 ARF and especially its short mitochondrial isoform, termed smARF, was also found to induce autophagy independent of p53 (20).

The activator protein-1 (AP-1) proteins are involved in most aspects of cellular proliferation, transformation, death, or survival, yet no data regarding their involvement in autophagy are available (21). c-Jun is an oncogene that positively regulates the cell cycle but can also modulate apoptosis, especially in cells exposed to genotoxic stresses and in the nervous system (21, 22). JunB is a cell cycle-regulated protein that is considered to antagonize c-Jun in proliferation and transformation and is not intensively involved in regulation of apoptosis (21, 23); however, recent studies implicate it in both promotion and repression of transformation (24). c-Jun and JunB regulate the expression of several important factors controlling proliferation, such as cyclin D (25), p16INK4a (26), and cyclin A (27). Jun proteins are regulated by JNK but are also induced by DNA damages that do not activate JNK (28). Whereas c-Jun regulation has been intensively studied, the full spectrum of stimuli regulating JunB activity is less understood. Recent reports have shown that JunB is regulated by two stimuli that may lead to autophagy (29, 30). This raised the possibility that JunB may be involved in regulation of autophagy. Here, we show that both c-Jun and JunB transcription factors inhibit autophagy induced by starvation. In addition, the expression of both is downregulated by starvation. The inhibition of autophagy requires their transcriptional activity and is independent of the mTOR pathway. Overexpression of JunB in HeLa cells increases cell death by starvation simultaneously with autophagy inhibition. Hence, we suggest that Jun proteins are inhibitors of autophagy whose repression is required for initiation of execution of the autophagic process.

Materials and Methods

Cells lines and treatments. Mouse embryo fibroblasts (MEF), HeLa, and HEK293 cells were grown at 37°C in an atmosphere of 5% CO₂ in high-glucose DMEM supplemented with 10% FCS. Earle's balanced salt solution (Biological Ind.) was used for starvation. The cells were treated with 100 μmol/L bafilomycin (LC Laboratories), 10 nmol/L rapamycin, 10 mmol/L 3-methyladenine (3-MA; Sigma). To ensure equal transfection efficiency in kinetic experiments, the cells were pool-transfected and then split to smaller plates, which were subjected to specific treatments.

Antibodies. JunB and p62/QSTM1 proteins were detected using N-17 or D-3 antibodies, respectively (Santa Cruz Biotechnology). Actin was detected using mouse anti-actin clone C-4 (MP Biomedicals). p-P70^{S6K} (Thr³⁸⁹), p-S6 (Ser²⁴⁰⁻²⁴⁴ or Ser^{235/236}), total P70^{S6K}, and total S6 levels were detected using polyclonal anti-phosphorylated p70 S6 kinase (Thr³⁸⁹) or anti-p-S6 and 49D7 or 5G10 monoclonal antibodies, respectively (Cell Signaling Technology). Cleaved caspase-3 was de-

tected using cleaved caspase-3 (Asp¹⁷⁵) Antibody (Cell Signaling Technology).

Electron microscopy. Electron microscopy was performed as previously described (31).

Immunofluorescence and detection of cell death. To detect autophagy in HEK293 and HeLa cells, the cells were plated on coverslips and transfected with LC-3 green fluorescent protein (GFP) plasmid (32) 24 h before treatment. After treatments the cells were fixed with 4% PFA. GFP was monitored using fluorescent microscopy, and the ratio between total LC3-expressing cells and cells expressing LC3 in distinct foci was determined. Images were visualized using an Olympus fluorescence microscope. To detect autophagy in MEFs, the cells were immunofluorescently stained for LC3 with a specific antibody (Sigma) after treatment with 100 μmol/L bafilomycin. Trypan blue exclusion, cleaved caspase-3, or nuclear fragmentation were monitored to determine apoptosis and cell death. Each experiment was repeated at least thrice. In each experiment at least 300 cells were counted.

DNA constructs. Mammalian expression vectors for JunB (28), JunB HD (33), smARF (20), and *shjunB* (34) were previously described. A pLKO.1-based, human *shjunB* expressing lentivirus was purchased from Open Biosystems (Oligo ID-TRCN0000014943). Lentiviruses, expressing shRNAs for both *junB* and HcRed or *junB* and beclin, were generated by substituting the puromycin resistance gene in pLKO.1 *shjunB* with the corresponding shRNAs sequences and the controlling H1-RNA promoters using BamHI and KpnI fragments digested from pSUPER expressing these sequences. These sites were also used to clone GFP-LC3 into pLKO.1 for generation of a lentivirus that expresses it. JunB DNA binding-deficient (DBD) mutant was generated by *in vitro* mutagenesis reactions to mutate R270, K271, R272, R285, and K286 to PEWEE using a commercial kit (QuikChange II site-directed mutagenesis kit, Stratagene). c-Jun DBD mutant and the JNK phosphorylation site mutants of c-Jun were previously described (34). All sequences used in the constructions of the vectors will be provided upon request.

Results

Starvation downregulates the expression of c-Jun and JunB. To explore possible involvement of AP-1 proteins in autophagy, we examined whether autophagy-inducing stimuli regulate their expression. As c-Jun affects cellular survival and proliferation in more than one way, our study focused primarily on the regulation and activity of JunB. Initially, changes in expression of JunB upon starvation, a well-known and most direct inducer of autophagy, were tested. As depicted in Fig. 1A and B, JunB expression is regulated by starvation. Immediately after starvation of MEFs, JunB expression is upregulated (Fig. 1A, top). However, after rapid and short-lasting induction of JunB, its expression declined below the basal level. The reduction in JunB protein level was also observed in immortalized fibroblasts (Fig. 1A, bottom). Examination of *junB* mRNA expression in MEFs revealed a 2-fold decrease in *junB* mRNA levels, which followed a short-lasting elevation (Fig. 1B). Similar repression

after starvation was also observed in c-Jun expression (Supplementary Fig. S1A and B). These results suggested that *junB* mRNA, as well as protein expression, is repressed following starvation and indicate that JunB is regulated at least at the level of mRNA expression; nevertheless, additional regulation at the posttranscriptional level cannot be excluded. To explore such effects, we transfected HEK293 cells with a plasmid expressing a minimal coding region of JunB controlled by SV40 promoter, starved the cells, and measured *junB* mRNA and protein expression simultaneously (Fig. 1C). Surprisingly, JunB protein expression was repressed without a similar decrease in *junB* mRNA levels, suggesting regulation at the posttranscriptional level.

A recent study suggested that the translation of JunB is regulated by mTOR (35). The activity of mTOR decreases following starvation (Fig. 1D; ref. 36); thus, JunB translation may be inhibited. To assess the contribution of mTOR inactivation to the repression of JunB expression, we determined the levels of transfected JunB, which unlike the endogenous one, is mostly regulated posttranscriptionally (Fig. 1C) in starved, rapamycin-treated, or rapamycin-treated and starved HEK293 cells. In all cases reduction in the levels of JunB was correlated with decreased activity of mTOR, measured by S6 phosphorylation (Fig. 1D), suggesting that inactivation of mTOR plays a role in the posttranscriptional regulation of JunB in this case as well.

Jun B is an inhibitor of autophagy. Regulation of JunB by starvation suggests a possible role in regulating autophagy. To test this possibility, we examined the effects of JunB on autophagy induced by different stimuli. Initially, we tested the effects of JunB on starvation-induced autophagy. Two different markers were examined, the distribution of LC3 in the starved cells and the steady-state levels of p62/SQSTM1 (37). Cotransfection of JunB into HEK293 cells together with GFP-LC3 reduced the number of cells displaying punctuated distribution of LC3 by ~50% and prevented the elimination of p62/SQSTM1 2 hours poststarvation (Fig. 2A). Similar results were also observed in HeLa cells (Supplementary Fig. 2). The presence or absence of bafilomycin A did not change the results (data not shown), suggesting that the effects of JunB are on the turn-on and not on the turn-off rate of autophagosomes turnover.

Another system used to determine the ability of JunB to inhibit autophagy was to examine its effects on smARF-induced autophagy. smARF is a very potent inducer of autophagy (20). JunB was added to cotransfected smARF and GFP-LC3, and the effects on autophagy and cellular survival were scored 24 and 48 hours later, respectively. As previously reported (20), smARF induced autophagy in almost 100% of the transfected cells. Surprisingly, despite equal levels of smARF expression in the presence of JunB (Supplementary Fig. S3), cotransfected JunB reduced the number of cells displaying punctuated distribution of LC3 by >50% and prevented the elimination of p62/SQSTM1 (Fig. 2B). Excessive autophagy by smARF leads to cell death (ref. 20; Fig. 2C). In fact, transfection of increased doses of smARF to HEK293 cells yields a correlative increase in cell death relative to transfection with vector alone (Fig. 2C), thus proving

that the death is smARF dependent. However, when JunB was cotransfected, smARF-induced death was prevented even after transfection with the highest dose of smARF (Fig. 2C). These results suggest that JunB can inhibit

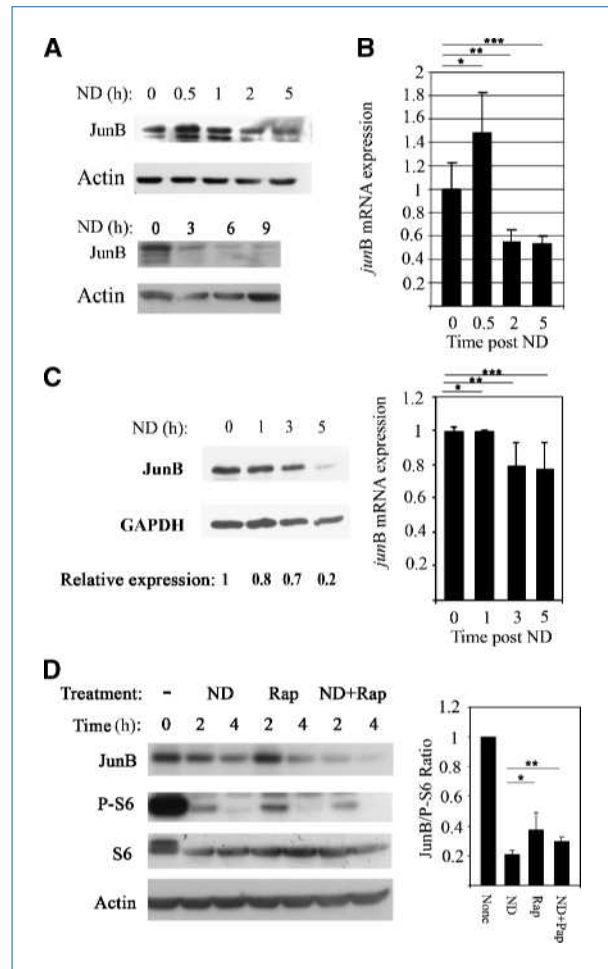


Figure 1. JunB is regulated by starvation. A, early passage MEFs (top) or immortalized fibroblasts (bottom) were starved and harvested at the indicated times. The expression of JunB protein was determined by immunoblotting using a specific antibody. Actin served as a loading control. ND, nutrient depletion. B, *junB* mRNA in MEFs was determined by quantitative real-time PCR (qRT-PCR). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were used for normalization. *, $P = 0.0193$; **, $P = 0.024$; ***, $P = 0.032$. C, HEK 293 cells were transfected with expression vector for JunB. Transfected cells were trypsinized, pooled, and replated 24 h after transfection to ensure equal transfection efficiency in each plate. The cells were starved 24 h later for the indicated times, and JunB protein (left) or mRNA (right) levels were determined by immunoblotting or qRT-PCR, respectively. GAPDH levels served as a loading control in both cases. The ratios between JunB and GAPDH whose quantities were measured by densitometry are presented as relative expression. *, $P = 0.72$; **, $P = 0.24$; ***, $P = 0.22$. D, HEK293 cells transfected with JunB were either starved, treated with 10 nmol/L rapamycin, or treated with both for the indicated times. The levels of JunB phosphorylated S6 and total S6 were determined by immunoblotting using specific antibodies. The ratio between JunB to phosphorylated S6 measured 2 h after treatment in three experiments are graphically depicted (right). *, $P = 0.09$; **, $P = 0.045$.

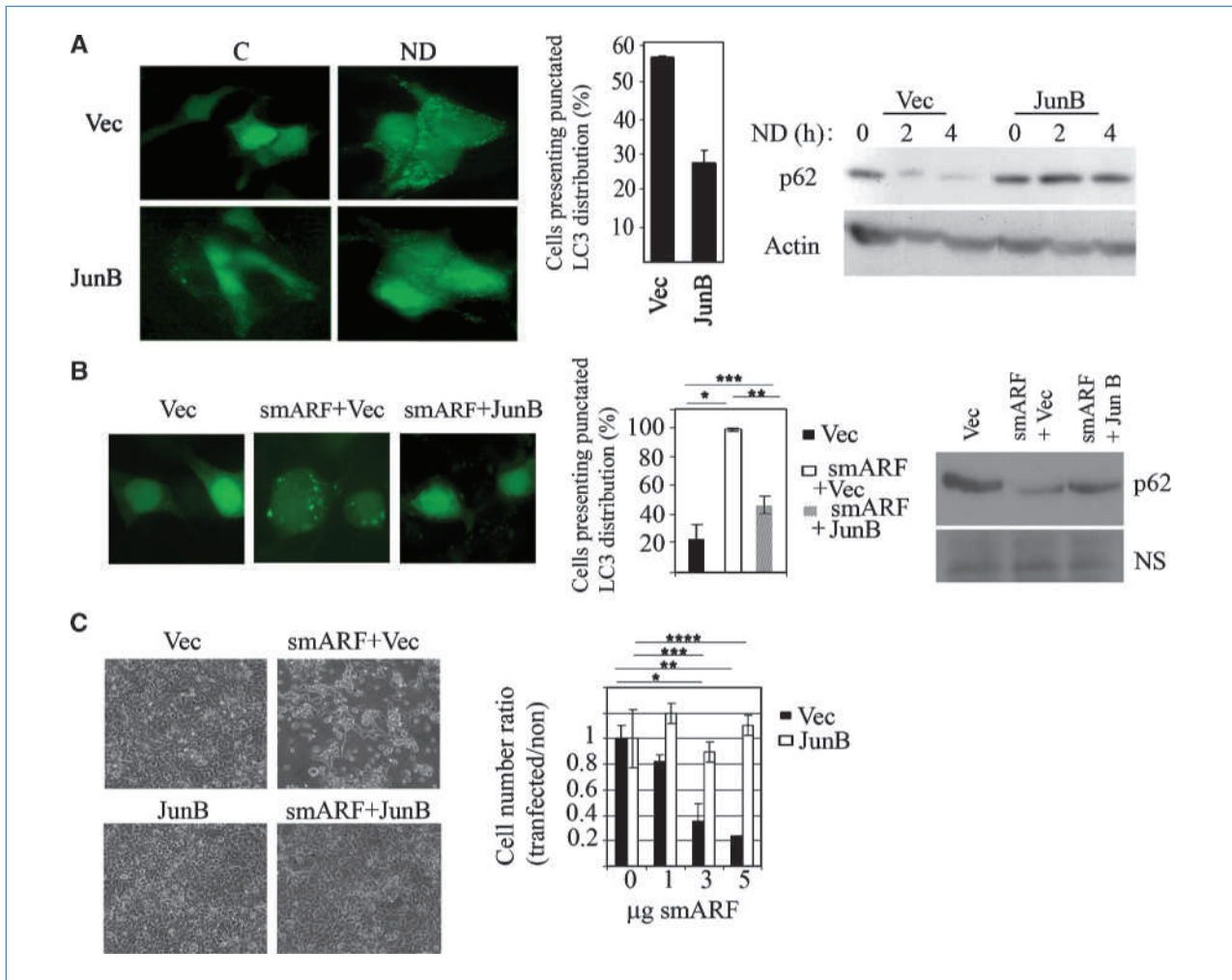


Figure 2. Overexpression of JunB inhibits autophagy. **A**, HEK293 cells were cotransfected with LC3-GFP and vector or LC3-GFP and JunB in a ratio of 1:30. LC3 distribution was detected 2 h after starvation using microscopy. The graph represents the percentage of cells displaying punctuated LC3 at this time point ($P = 0.0040$). At least 300 cells were counted for each experiment, and every experiment was repeated at least thrice. Endogenous p62 levels (right) were measured by immunoblotting using a specific antibody. Actin served as a loading control. **B**, HEK293 cells were transfected with 2 μg of smARF, 5 μg of vector or junB, and 0.1 μg of LC3-GFP, as indicated. The distribution of LC3 was monitored 24 h later by microscopy (left) and graphically presented (*, $P = 0.007$; **, $P = 0.098$; ***, $P = 0.043$). Endogenous p62 levels (right) were measured by immunoblotting using a specific antibody. NS, nonspecific band. **C**, HEK293 cells transfected with the indicated plasmids (3 μg of smARF and 5 μg of junB) and photographed 48 h after transfection. Cell death was measured in HEK293 cells transfected with the indicated doses of smARF and 5 μg of either vector or JunB by dye exclusion 48 h after transfection and graphically presented (right). *, $P = 0.032$; **, $P = 0.009$; ***, $P = 0.038$; ****, $P = 0.035$.

autophagy and cell death induced by overexpression of smARF as well.

The results described above are based on JunB overexpression. To further establish our findings, we examined the effect of JunB downregulation on autophagy. As the levels of JunB are low in most tumor-derived human cell lines, MEFs were infected with retroviruses expressing shRNA for JunB and selected. Stable cultures expressing <30% to 35% JunB compared with JunB expression in cells infected with shLacZ-expressing retrovirus could not be generated. We therefore determined the effects of JunB knockdown after semistable infection, i.e., the cells were infected and examined 3 days after selection. JunB shRNA efficiently repressed

JunB expression (Fig. 3A). When the cells were exposed to bafilomycin A1 and immunostained with specific antibody against LC3, distinct foci characteristics of autophagosome formation were apparent in shJunB-expressing cells (Fig. 3B). Electron microscopy revealed the presence of autophagosomes in shJunB-expressing cells as well (Fig. 3C). Determination of the effects of shJunB on the survival/proliferation of MEFs also revealed autophagy-dependent effects. The number of MEFs expressing shLacZ declined up to 48 hours after selection, a time point at which noninfected cells die due to antibiotic selection, and afterward started to increase again, whereas the reduction in cell number of MEFs expressing shJunB was greater and no increase was observed 72 hours

after selection (Fig. 3D, left). In addition, treatment of MEFs expressing shjunB with the autophagy inhibitor 3-MA that efficiently inhibits autophagy in this system (Supplementary Fig. S4), simultaneously with the beginning of puromycin selection, enhanced their survival up to a level equivalent to that of the control MEFs, unlike the low basal toxicity that slightly reduced the survival of shLacZ-expressing MEFs (Fig. 3D, right). This result proves that the reduction in the survival of MEFs expressing shjunB is caused by excessive autophagy. Infection of human foreskin fibroblasts with lentivirus expressing shjunB targeting the human JunB resulted in an increase in autophagy as determined by LC3 distribution, LC3II formation, and p62 steady-state levels (Supplementary Fig. S5). In this case however, the cells displayed the autophagic markers but did not die. Collectively, these results support the notion that JunB inhibits autophagy induced by different factors.

Inhibition of autophagy by Jun B is not mediated by mTOR regulation. mTOR is a major inhibitor of autophagy (reviewed by ref. 36) and therefore serves as a target for regulation by several factors affecting autophagy, including the tumor suppressor p53 (19). We tested if the inhibition

of autophagy by JunB is mediated by regulation of mTOR activity. Phosphorylation of the direct mTOR substrate p70^{S6K} and a further downstream substrate in the mTOR pathway, S6, was measured at different times after starvation of vector or JunB-transfected HEK293 cells. As depicted in Fig. 4A, a small elevation in the basal activity of mTOR was observed, but JunB did not prevent the predicted decline in mTOR activity measured by the phosphorylation of its substrates after starvation. To directly determine if effects on mTOR activity play a substantial part in the ability of JunB to prevent autophagy we examined the relationship between JunB and mTOR in two experimental systems. First we examined if JunB suppresses autophagy even when the mTOR pathway is artificially silenced. HEK293 cells transfected with vector or JunB were starved or treated with rapamycin, and the effects of JunB on autophagy induced by each of the stimuli were determined (Fig. 4B, left). The phosphorylation of S6 was concomitantly measured to test mTOR activity (Fig. 4B, right). Both factors, starvation and rapamycin treatment, increased the number of cells displaying punctuated LC3 by >40% and 20%, respectively, and reduced the activity of mTOR. In fact, rapamycin totally

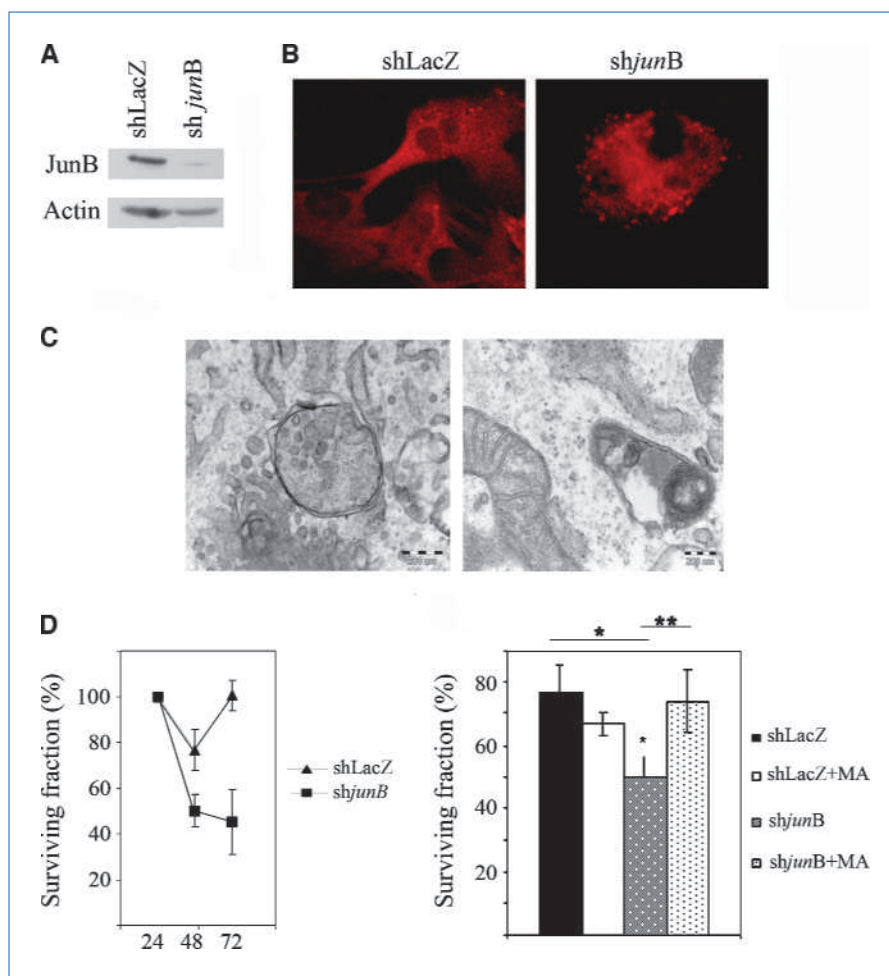


Figure 3. Downregulation of JunB expression induces autophagy in MEFs. Early passage MEFs (p2) were infected with retroviruses expressing shRNA for junB or LacZ. The cells were harvested 48 h after selection to determine JunB levels by immunoblotting (A), treated with 100 μ mol/L bafilomycin A for 2 h, fixed, and immunofluorescently stained using antibody specific for LC3 (B), or counterstained and subjected to electron microscopy (C). D, shLacZ- or shjunB-transduced MEFs were divided concurrently with the addition of the antibiotics (1 μ g/mL puromycin) and counted at the indicated times. To eliminate possible effects of the seeding efficiency of each cell culture, the number of cells 24 h after seeding, and not the number of plated cells, was considered to be 100%, and the changes in cell numbers were compared with this point (left). The autophagy inhibitor 3-MA was added at a concentration of 10 mmol/L simultaneously with the puromycin selection. The cells were counted 48 h later (right). *, $P = 0.0440$; **, $P = 0.0448$.

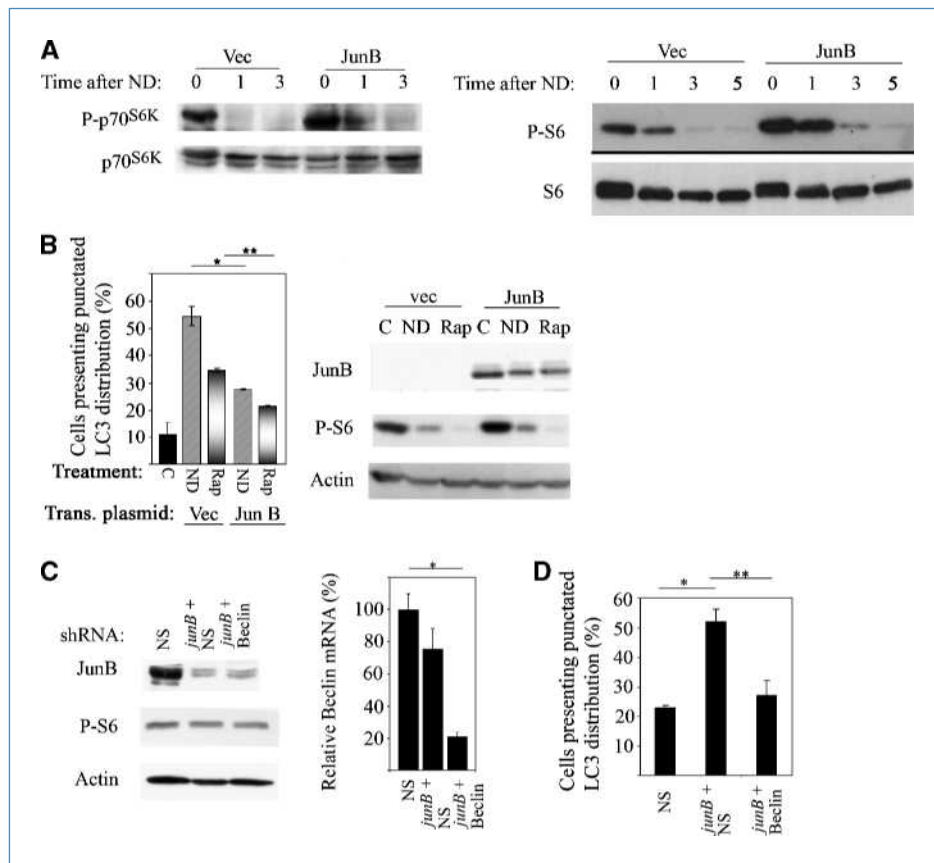


Figure 4. JunB inhibits autophagy in an mTOR-independent manner. HEK293 cells were transfected with vector or JunB expressing plasmid and starved for the indicated times (h). A, the levels of phosphorylated p70^{S6K} (left) and phosphorylated S6 (right), as well as the total levels of the proteins, were detected by specific antibodies. B, HEK293 cells transfected as in A were either starved (ND) or treated with rapamycin (Rap) for 3 h, and the levels of autophagy (left) and S6 phosphorylation (right) were detected by determination of LC3 distribution or by immunoblotting using specific antibodies, respectively. *, $P = 0.0233$; **, $P = 0.0015$. C, HeLa cells were cotransduced with mixtures of lentiviruses expressing GFP-LC3 and the indicated shRNAs (1:5 ratios). Five days after infection the cells were either harvested to determine JunB and phosphorylated S6 expression by immunoblotting (C, left), beclin expression by quantitative PCR (C, right), or microscopically examined to determine percentage of cells presenting punctuated LC3 distribution (D). C, actin and GAPDH served as loading controls for immunoblotting and quantitative PCR, respectively. NS, non specific. $P = 0.0003$. D, *, $P = 0.0049$; **, $P = 0.0039$.

abolished S6 phosphorylation. Despite this fact, JunB was able to inhibit autophagy induced by either starvation or rapamycin by 1.9-fold and 1.6-fold, respectively, thus suggesting that the inhibition of autophagy is independent of the effects on the mTOR pathway. In an additional experiment designed to test the relationship between JunB and mTOR activity, we examined whether a correlated change in mTOR activity will be observed when autophagy driven by JunB knockdown is induced. HeLa cells were cotransduced with different mixtures of lentiviruses. Each mixture contained a lentivirus expressing GFP-LC3 and a virus expressing either shRNA targeting HcRed, referred to hereafter as nonspecific shRNA, and used as a control, a virus coexpressing nonspecific and *junB* shRNAs or a virus expressing shRNAs for *junB* and Beclin. Five days postinfection the effect on autophagy was determined by scoring for LC3 distribution, and the effect on mTOR activity was determined by detection of S6 phosphorylation. As predicted, *junB*

shRNA efficiently repressed JunB expression (Fig. 4C) and increased the number of cells displaying punctuated LC3 distribution (Fig. 4D). Repression of beclin expression concomitantly with that of *junB*, by specific shRNA (Fig. 4C, right), inhibited the autophagy induced by JunB knockdown (Fig. 4D). However, no correlated reduction in mTOR activity was observed in cultures presenting elevated numbers of cells with punctuated LC3 distribution due to *junB* knockdown (Fig. 4C, left). Hence, these results further support the notion that inhibition of autophagy by JunB is independent or affects components downstream of the mTOR pathway.

AP-1 dependent transcription is required for the inhibition of autophagy. JunB is a transcription factor that homodimerizes or heterodimerizes to activate gene transcription (21, 24, 38). Therefore, we tested if the effects of JunB on the prevention of autophagy are mediated by its ability to regulate gene transcription. To examine if JunB cooperates

with other AP-1 partners in the inhibition of autophagy, we measured the inhibition of starvation-induced autophagy by a JunB mutant incapable of heterodimerizing due to substitution of its naturally occurring dimerization domain with a heterologous homodimerization domain from the EBV transcription factor EB1, HD JunB, also known as JunBeb1 (33). The starvation-induced autophagy in HEK293 cells expressing this mutant was inhibited by 25%, whereas wild-type (wt) JunB repressed autophagy by 72% when equally expressed (Supplementary Fig. S6). This result suggests that JunB probably cooperates with other AP-1 proteins to inhibit autophagy. To determine which other AP-1 proteins are capable of inhibiting autophagy, we monitored the LC3 distribution in starved HEK293 cells after transfection of c-Jun, JunB or JunD, c-Fos, or Fra-1. As depicted in Fig. 5A, c-Jun, but not JunD, c-Fos, or Fra-1 may also reduce the number of cells displaying punctuated LC3 in addition to JunB. To assess the importance of c-Jun and JunB transcriptional activity for autophagy inhibition, we generated a JunB mutant that is incapable of binding DNA by mutating five basic residues

(R270/P, K271/E, R272/W, R285/E, R286/E) residing within its DNA binding domain and used a previously described c-Jun DNA binding-deficient mutant, in which two basic residues, Arg²⁷² and Lys²⁷³, were replaced with two glutamic acids (34). The ability of the mutants to inhibit starvation-induced autophagy in HEK293 cells was compared with that of wt JunB. As depicted in Fig. 5B, increasing doses of wt JunB reduced starvation-induced autophagy in a dose-dependent manner, whereas the DNA DBD (JunB) was not able to do so. Similar results were obtained with a DBD c-Jun (Fig. 5C). In fact, in this case the mutant increased the starvation-induced autophagy, suggesting a dominant negative activity. These results suggested that the DNA binding and transcriptional activities of JunB and c-Jun are essential for the inhibition of autophagy. Next, we examined whether c-Jun-dependent inhibition of autophagy is mediated by JNK phosphorylation. To that end we compared the ability of wt c-Jun and two JNK phosphorylation mutants, 63/73Ala and 91/93Ala, to inhibit starvation-induced autophagy (Fig. 5D). As depicted in Fig. 5D, the mutants inhibited autophagy

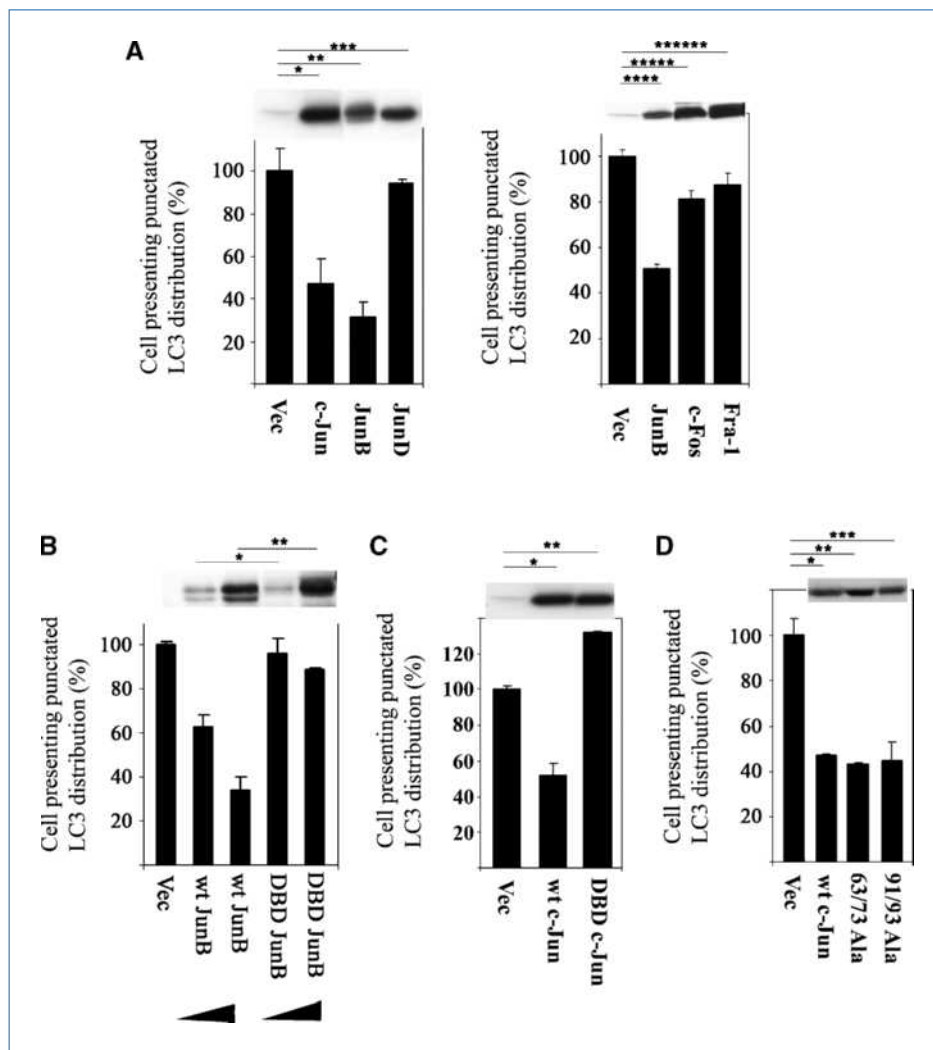
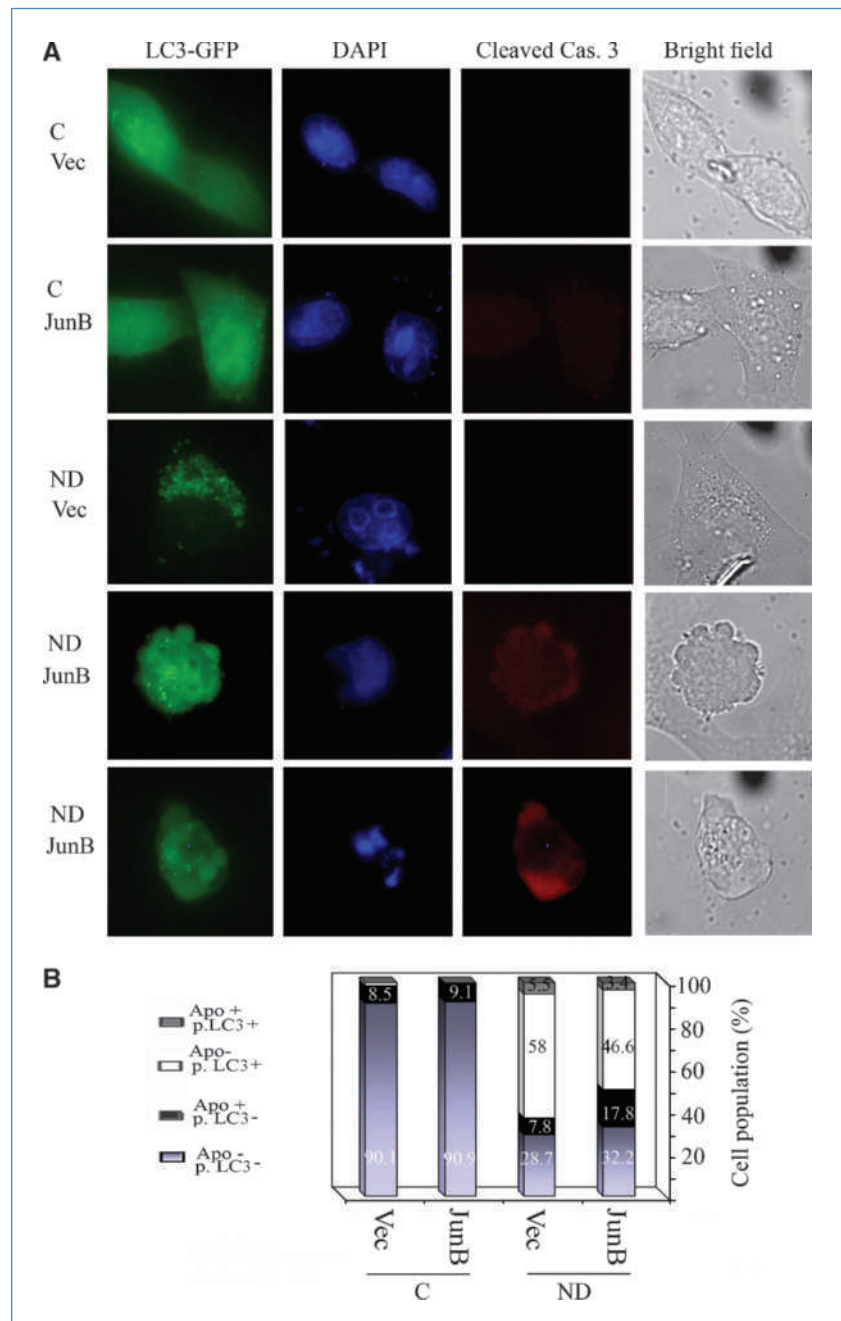


Figure 5. The transcriptional activity of JunB and c-Jun is required for the inhibition of autophagy. A, C, D, 2 μ g of the indicated plasmids were cotransfected together with 0.1 μ g LC3-GFP into HEK293 cells. Transfected cells were starved for 3 h, and the fraction of cells displaying punctuated LC3 distribution from the entire transfected population was determined. The fraction observed in vector-transfected cells, 3 h after starvation, was considered as 100%, and all other values were related to this value. The levels of transfected proteins were determined using anti-HA antibody, recognizing HA-tag fused to all of the indicated proteins. B, HEK293 cells were cotransfected with 0.1 μ g of LC3-GFP and 2 μ g of vector or with either 1 or 2 μ g of wt or DBD JunB expression vectors. LC3 distribution was monitored 3 h poststarvation. The levels of transfected JunB were determined by immunoblotting using the HA antibody as well. A, *, $P = 0.04$; **, $P = 0.024$; ***, $P = 0.057$; ****, $P = 0.00023$; *****, $P = 0.00083$; *****, $P = 0.016$. B, *, $P = 0.032$; **, $P = 0.044$. C, *, $P = 0.039$; **, $P = 0.044$. D, *, $P = 0.0064$; **, $P = 0.0056$; ***, $P = 0.013$.

Figure 6. JunB inhibits starvation-induced autophagy and enhances apoptosis. HeLa cells were cotransfected with 0.2 μ g LC3-GFP and 2 μ g of either vector or JunB. The cells were starved for 6 h stained with 4',6-diamidino-2-phenylindole (DAPI) and immunostained for cleaved caspase-3. Fluorescent images, as well as bright field images, were photographed to determine the percentage of nonapoptotic, nonautophagic, apoptotic, autophagic, or apoptotic and autophagic cells from the entire population of transfected cells. A, representing images. B, graphic presentation. Apo, apoptosis; p.LC3, punctuated LC3.



comparable with the wt protein, proving that the inhibition is JNK independent.

JunB-dependent inhibition of starvation-induced autophagy leads to cell death. Starvation of HeLa cells rapidly induces autophagy. Inhibition of autophagy under these conditions will result in enhanced cell death (39, 40). Therefore, our results suggested that overexpression of JunB will increase cell death as a result of autophagy inhibition. To test this point, HeLa cells were cotransfected with vector or JunB and GFP-LC3 and starved, and punctuation of

LC3 as well as three parameters to detect apoptosis, nuclear condensation and fragmentation, caspase 3 cleavage, and cellular morphology were measured 6 hours after starvation. At this time point the cellular population may be divided to several subpopulation. Cells that do not exhibit characteristics of either autophagy or apoptosis, cells exhibiting punctuated LC3, cells undergoing apoptosis, or cells exhibiting markers for apoptosis and autophagy are exhibited in Fig. 6. Interestingly, ectopic expression of JunB resulted in reduction of the subpopulation of cells exhibiting the

autophagic distribution of LC3 by ~12% concomitantly with a 10% increase (more than doubling) in the population undergoing apoptotic cell death (Fig. 6B). These results suggest that the unregulated expression of JunB may enhance cell death by the inhibition of autophagy.

Discussion

AP-1 transcription factor is one of the immediate early factors that responds to mitogenic or stress signals (21). Involvement of AP-1 proteins in most aspects of cellular proliferation, death, or survival has been extensively studied (21), yet the involvement of AP-1 proteins in autophagy remains unknown. Here, we show that c-Jun and Jun B inhibit autophagy caused by starvation, smARF overexpression, or even rapamycin treatment. Hence, we suggest that the Jun proteins are inhibitors of autophagy. The link presented in this study between the acute repression of JunB expression (obtained in this study by shRNA-mediated knockdown) and the induction of autophagy suggests an essential role for its repression in starvation conditions. However, the fact that, shortly after starvation, JunB is transiently induced also suggests that JunB regulates cellular homeostasis by preventing autophagy in cases of transient starvation. Nevertheless, prolonged exposure to autophagy-inducing stimuli eventually results in autophagy due to downregulation of JunB, resulting from repression of JunB mRNA expression and additional posttranscriptional effects. Regulation of at least two factors during autophagy may affect JunB expression. NF- κ B is a regulator of JunB transcription (29). NF- κ B's upstream activator IKK is inactivated by autophagy (41, 42). Thus, it is conceivable that inactivation of NF- κ B results in downregulation of JunB transcription. Conversely, identification of the ability of JunB to inhibit autophagy may provide some theoretical explanation for the ability of NF- κ B to inhibit starvation-induced and other types of autophagy (43, 44). The posttranscriptional downregulation of JunB following starvation is at least partially dependent on the activity of mTOR as previously reported by others (35); hence, the significant decrease in mTOR activity following starvation is predicted to reduce JunB expression.

Overexpression experiments suggest that the repression of JunB expression is required to allow timely and effective autophagy, as elevated levels prevent the rapid autophagic response observed 2 hours after starvation in vector-transfected HEK293 and HeLa cells. In addition, as previously observed by impairment of essential components of the autophagic machinery (39), JunB-dependent

inhibition of autophagy results in enhancement of apoptotic cell death. Our results may also suggest that the prolonged induction of c-Jun and JunB after exposure of cells to environmental carcinogens, such as UV radiation (34), may inhibit autophagy expected theoretically following activation of JNK and induction of p53 (17, 18).

Interestingly, specificity in the ability to inhibit starvation-induced autophagy is observed among the AP-1 protein tested, as only overexpression of c-Jun and JunB inhibited autophagy whereas overexpression of JunD, c-Fos, and Fra-1 did not. In both cases transcriptional activity of the Jun proteins is required. This suggests that AP-1-dependent target activation is required. Moreover, c-Jun incapable of binding DNA seemed to serve as dominant negative, as it increased the number of cells presenting punctuated LC3 as well as the average size of LC3 foci. In agreement with the fact that JNK activity induces autophagy, c-Jun inhibits autophagy in a JNK-independent manner.

Collectively, we show that JunB and c-Jun are potent inhibitors of autophagy whose expression is regulated by autophagy-inducing stimuli. The biological activity of JunB in regulation of proliferation and tumorigenesis is quite elusive, supporting each in some cases and inhibiting in others (24). These opposing effects are attributed to regulation of cell cycle-controlling genes. We suggest that the ability of JunB to modulate autophagy, especially under conditions of metabolic stress, is an additional factor affecting the consequences of its deregulated expression. Furthermore, induction of autophagy by downregulation of JunB may also shed new light on the activity of AP-1 in maintaining normal cellular homeostasis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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