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C-Jun N-terminal kinases are required for oncolytic adenovirus-mediated autophagy

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Abstract

Oncolytic adenoviruses, such as Delta-24-RGD, are replication-competent viruses that are genetically engineered to induce selective cancer cell lysis. In cancer cells, Delta-24-RGD induces massive autophagy, which is required for efficient cell lysis and adenoviral spread. Understanding the cellular mechanisms underlying the regulation of autophagy in cells treated with oncolytic adenoviruses may provide new avenues to improve the therapeutic effect. In this work, we showed that cancer cells infected with Delta-24-RGD undergo autophagy despite the concurrent activation of the AKT/mTOR pathway. Moreover, adenovirus replication induced sustained activation of JNK proteins *in vitro*. ERK1/1 phosphorylation remained unchanged during adenoviral infection, suggesting specificity of JNK activation. Using genetic ablation and pharmacological inactivation of JNK, we unequivocally demonstrated that cells infected with Delta-24-RGD required JNK activation. Thus, genetic co-ablation of *JNK1* and *JNK2* genes or inhibition of JNK kinase function rendered Delta-24-RGD-treated cells resistant to autophagy. Accordingly, JNK activation induced phosphorylation of Bcl-2 and prevented the formation of Bcl-2/Beclin 1 autophagy suppressor complexes. Using an orthotopic model of human glioma xenograft, we showed that treatment with Delta-24-RGD induced phosphorylation and nuclear translocation of JNK, as well as phosphorylation of Bcl-2. Collectively, our data identified JNK proteins as an essential mechanistic link between Delta-24-RGD infection and autophagy in cancer cells. Activation of JNK without inactivation of the AKT/mTOR pathway constitutes a distinct molecular signature of autophagy regulation that differentiates Delta-24-RGD adenovirus from the mechanism used by other oncolytic viruses to induce autophagy and provides a new rationale for the combination of oncolytic viruses and chemotherapy.

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Keywords

Oncolytic adenovirus; Autophagy; JNK; Bcl-2; Delta-24

INTRODUCTION

Tumor-selective oncolytic adenoviruses are being tested for the treatment of cancer.¹ We have previously characterized the anticancer effect of Delta-24-RGD (Δ 24RGD) in orthotopic models of glioma.² In addition, we have reported that Delta-24-RGD induces autophagy in cancer cells^{3, 4} and that adenovirus-induced lysis of cancer cells depends on the induction of autophagy.⁵ Understanding the cellular mechanisms underlying the regulation of autophagy in cancer cells infected with oncolytic adenovirus may provide new avenues to improve the potency or selectivity of their therapeutic effect.

The current paradigm for the modulation of autophagy in mammalian cells is based on the molecular changes produced by starvation.⁶⁻⁸ In cells that are starving, a nutrient-sensing mechanism regulated by the balance between the functions of AMP-activated protein kinase (AMPK) (positive regulator) and the mammalian target of rapamycin (mTOR) (negative regulator) governs autophagy.⁶⁻⁸ Consistent with a potential universal relevance, the AMPK/mTOR pathway is not only key during starvation but also is a common target for several types of viruses to modulate autophagy in the host cells.^{9, 10}

In addition to mTOR, the c-Jun N-terminal kinase (JNK) signal transduction pathway has been implicated in the regulation of autophagy. However, the role of these kinases in autophagy is controversial since they have been involved in both the activation¹¹ and suppression¹² of autophagy. Moreover, the function of JNK in virus-induced autophagy has not been examined yet.

The adaptor protein Beclin 1 exerts autophagy-modulating functions and is located downstream of several pathways.¹³ Beclin 1 is a Bcl-2 homology 3-only protein¹³ and was first identified as a Bcl-2 binding protein.¹⁴ Anti-autophagic function of Bcl-2 is mediated by the sequestering of Beclin 1, preventing its incorporation into the pro-autophagy PI3KC3 complexes.^{11, 14} On the other hand, Bcl-2 phosphorylation blocks the formation of Bcl-2/Beclin 1 complexes and in turn activates autophagy.^{11, 14} Interestingly, in starving cells, the JNK1 isoform, but not any of the other isoforms, phosphorylates Bcl-2, preventing the formation of Bcl-2/Beclin 1 heterodimers.¹¹ Currently, no information exists about the potential role of JNK isoforms in the regulation of Beclin 1 and autophagy in cancer cells treated with oncolytic adenoviruses.

RESULTS AND DISCUSSION

Oncolytic viruses are becoming one of the most promising anticancer agents.¹ Although their safety has already been demonstrated in clinical trials,¹⁵ enhanced potency seems to be required to induce optimal therapeutic effect in human patients. Delta-24-RGD oncolytic adenovirus² induces cancer cell lysis by activating autophagy pathways during the replication cycle.^{3, 4} Therefore, pharmacological modulation of autophagy may result in an

increased oncolytic effect. However, the molecular events that modulate autophagy in cancer cells infected by tumor-selective adenoviruses remain poorly understood.

In this study, we first asked whether the canonical regulatory pathway of autophagy⁶⁻⁸ was activated during adenoviral infection. In contrast to observations obtained with starving cells, MRC5 lung fibroblasts infected with wild-type adenovirus (AdWT) exhibited negligible variations in AMPK α 1 and AMPK α 2 phosphorylation. Moreover, we showed an increase in AKT phosphorylation,⁶⁻⁸ strongly suggesting that the mechanisms regulating autophagy in adenovirus-infected cells were clearly different from those regulating autophagy in cell starvation. Activation of AKT started within 16 h of infection (22-fold) and was sustained 48 h after infection (26-fold) (Figure 1a, Supplementary Figure 1, and Supplementary Table 1). In agreement with AKT activation, the levels of phosphorylated mTOR increased with time, peaking at a level 31 times higher than the basal level within 48 h after infection (Figure 1a, Supplementary Figure 1, and Supplementary Table 1). Confirming these data, Delta-24-RGD caused a similar increase in AKT phosphorylation in U87 MG glioma cells (Figure 1b). Further suggesting that the AMPK/AKT pathway was not relevant in adenovirus-induced autophagy, AdWT-infected cells underwent autophagy irrespective of *AMPK α 1* (Figure 1c) or *tuberous sclerosis protein 2 (TSC2)* gene status, whose protein product is phosphorylated and inhibited by AKT and suppresses mTOR signaling (Figure 1d).^{6,7} Since the status of AMPK was not relevant for adenovirus-induced autophagy and since we did not observe consistent activation of ERK proteins, our results indicate that oncolytic adenoviruses do not trigger autophagy via activation of the noncanonical AMPK-ERK-TSC pathway.¹⁶

Delta-24-RGD infection activates pathways differ from those discovered in studies performed with other viruses. Thus, activation of AMPK is described in cells expressing the SV40 ST antigen,⁹ as well as in cells infected with vesicula stomatis virus.¹⁰ Furthermore, other viruses, including human cytomegalovirus, target the mTOR signaling pathway to inactivate autophagy.¹⁷ Meanwhile, SV40ST, vesicula stomatis virus, and human cytomegalovirus constitute paradigms of the subversion of canonical, nutrient-sensing, autophagy regulatory pathways.⁶⁻⁸ Of interest, activation of AKT in cancer-infected cells is essential to favor the synthesis of adenoviral versus cellular proteins.^{18,19} Therefore induction of autophagy should be initiated and maintained by other mechanisms that do not impede the efficient replication of the virus. In this regard, we observed that in addition to the AKT/mTOR pathway, adenovirus infection triggered a progressive increase in the phosphorylation of JNK proteins. Specifically, within 16 h of infection, we documented an ~3.5 times higher level of phosphorylated JNK and an ~4.5 times higher level of c-Jun (Figure 2a, Supplementary Figure 2a). The progressive increase in the activation of JNK was remarkable, and we observed a 35-fold increase in phosphorylated JNK within 48 h of adenoviral infection (Figure 2a). We could detect no significant modification in the levels of ERK1/2 phosphorylation (Figure 2a), the activation of which plays a role in the regulation of autophagy by Epstein-Barr virus²⁰; these finding suggest that the activation of JNK may be specific.

We confirmed that the phosphorylation of JNK and c-Jun in host cells infected a panel of cancer cell lines with AdWT or Delta-24-RGD (Figures 2b and c, Supplementary Figure

2b). Phosphorylation of JNK and c-Jun in HeLa cells was evidenced within 6 h of infection and showed a progressive increase over time (Figure 2b). As expected, Delta-24-RGD adenovirus, which was capable of infecting U87 MG cells more efficiently than AdWT,^{2, 21} induced changes in the phosphorylation of JNK and c-Jun to a greater extent in U87 MG cells than did AdWT at similar doses (Supplementary Figure 2b).

We next examined the phosphorylation of each JNK isoform upon adenovirus infection and observed that JNK1 and JNK2 were highly phosphorylated during adenoviral replication (25- and 16-fold increase, respectively); however, phospho-JNK3 levels remained unchanged up to 36 h after infection (Supplementary Figures 2c and d; Supplementary Table 2).

To determine whether changes in JNK phosphorylation basal levels had any physiological relevance, we examined the phosphorylation status of JNK in glioma xenografts implanted intracranially and treated with Delta-24-RGD² (Figure 2d). Immunofluorescence analyses showed that JNK was phosphorylated in the majority of adenovirus-infected cells and co-localized with adenoviral structural proteins, an indicator of adenoviral replication.² Of note, we observed that in infected cells, the pJNK was predominantly nuclear, strongly suggesting that JNK was active and functional (Figure 2d). Thus, the immunofluorescence analyses showed the temporal correlation of pJNK and adenoviral replication *in vivo*.

Activation of JNK and AKT, together with the lack of AMPK activation, mechanistically set apart adenovirus-mediated autophagy from starvation-induced autophagy⁶⁻⁸ and demonstrated that autophagy in adenovirus-infected cells is distinctly regulated and differs from the mTOR targeting observed in cells infected by other viruses. Thus, the trigger of autophagy in infected cancer cells is not mediated by energy sensors, as in starving cells, but more likely involves the aberrant intracellular production of adenoviral proteins, resulting in endoplasmic reticulum stress and subsequent activation of JNK proteins.²²⁻²⁴

Beclin1 and Bcl-2 complexes are common targets for virus proteins capable of inhibiting²⁵ or activating autophagy.⁴ In this study, we showed that Bcl-2 phosphorylation levels consistently increased after infection and that progressive Bcl-2 phosphorylation was coincident in time with formation of autophagosomes, as assessed by LC3 status, and progressive autophagy (Figure 3a and Supplementary Figures 3a and b). We sought to validate these *in vitro* data with use of our *in vivo* xenograft model of U-87 MG human glioma tumors (Figure 3b). In these tumors, phospho-Bcl-2 protein co-localized with adenoviral proteins and was observed only in infected cells. Unlike phospho-JNK, phospho-Bcl-2 expression was restricted to the cytoplasm. Collectively, these studies showed for the first time that in human tumors infected with adenovirus, the time of JNK activation, Bcl-2 phosphorylation, and adenovirus replication was coincidental.

Next we asked whether functionally active JNK was required for the phosphorylation of Bcl-2 during adenovirus infection. To this end, we planned to inhibit JNK function with SP600125, an ATP-competitive inhibitor of the three isoforms of JNK.²⁶ Pretreatment of A549 lung cancer cells with SP600125 was sufficient to drastically inhibit the progressive increase in Bcl-2 phosphorylation after adenovirus infection (Figure 3c). To corroborate

these data, we examined the status of Bcl-2 phosphorylation and the capability of Bcl-2 to interact with Beclin1 protein in isogenic MEFs lacking *JNK1* and/or *JNK2* genes after adenovirus infection. Whereas adenoviral infection of wild-type-*JNK* MEFs (*JNK wt*) resulted in increased Bcl-2 phosphorylation, co-deletion of *JNK1* and *JNK2* (*JNK1/2-/-*) genes prevented the phosphorylation of Bcl-2 (Figure 3d). In agreement with these data, adenoviral infection of *JNK wt*, *JNK1-/-*, and *JNK2-/-* MEFs suppressed the formation of Bcl-2/Beclin 1 heterodimers (Figure 3e) but had no effect on the formation of these complexes in *JNK1/2-/-* MEFs, as demonstrated by coimmunoprecipitation (Figure 3e). Therefore, inactivation of JNK 1 or JNK2 was not sufficient to inhibit the interactions at the protein level between Bcl-2 and Beclin 1, and combined inactivation of both *JNK1* and *JNK2* genes was required to release Bcl-2 from the Beclin 1 inhibitory complexes. These data suggested no requirement for JNK3 in the examined cell systems and were in agreement with the minimum changes detected in phospho-JNK3 levels after adenovirus infection (Supplementary Figure 2c); furthermore, these data agreed with the fact that expression of functionally active JNK3 is restricted to neurons and cardiomyocytes.^{27, 28}

To confirm that inhibition of JNK-mediated regulation of Bcl-2 phosphorylation resulted in blockade of autophagy, we pretreated A549 cells with SP600125 followed by adenoviral infection for 48 h. We showed that upon adenovirus infection, JNK activation was coincident with the formation of autophagosomes, and thus JNK-selective inhibition blocked the conversion of LC3-I to LC3-II (Figure 4a). We then evaluated the expression of p62 (p62/SQSTM1; sequestosome) to examine the completion of the autophagy flux.²⁹ Upon infection, and as expected, we observed a decrease in the p62 protein levels in A549 cells infected with adenoviruses (Figure 4a).⁵ However, exclusive inhibition of JNK prevented the degradation of p62 (Figure 4a). In agreement with these data, there was a significant reduction in the percentage of U87 MG cells transfected with the EGFP-LC3 and treated with SP600125 before infection with Delta-24-RGD, displaying the characteristic autophagy-related cytoplasmic foci (Figure 4b). The levels of p62 protein also decreased markedly after infection of *JNK wt*, *JNK1-/-*, or *JNK2-/-* MEFs (Figure 4c). However, co-deletion of both isoforms of *JNK* prevented degradation of p62 in adenovirus-infected cells (Figure 4c). The role of the two isoforms of JNK in adenoviral-induced autophagy was further demonstrated with use of electron microscopy. Cells subjected to the co-deletion of *JNK1* and *JNK2* did not exhibit the accumulation of characteristic double-membrane-bound cytoplasmic vacuoles,³⁰ whereas numerous vacuoles, occupying vast areas of the cytoplasm, were observed in *JNK wt*, *JNK1-/-*, and *JNK2-/-* cells after adenoviral infection (Supplementary Figures 4a and b). We concluded that *JNK1* and *JNK2* encoded proteins displayed overlapping or complementary functions in the regulation of adenovirus-mediated autophagy. Our data are in agreement with the common tenet that JNK1 and JNK2 exert similar functions in many cellular scenarios³¹ but differ from the roles of JNK 1 and JNK2 in other settings of stress-induced autophagy, in which JNK1 isoform, but not JNK2 isoform, is involved in the regulatory mechanism of autophagy.¹¹

Of interest, despite the fact that c-Jun phosphorylation increased during adenoviral replication (Figures 2b and c), genetic inactivation of *c-Jun* expression was not sufficient to prevent p62 degradation in adenovirus-infected MEF cells, suggesting that JNK-dependent

regulation of autophagy may be independent of c-Jun or that function of c-Jun in the regulation of adenovirus-mediated autophagy overlaps with other downstream targets of JNK (Figure 4d).

In summary, we show that the pathways that modulate autophagy in cells infected with adenoviruses are distinctly different from the canonical signaling involving the inactivation of the PI3K/AKT/mTOR pathway that modulates starvation-mediated autophagy. Actually, our data showed a dramatic activation of this pathway, which is key for the synthesis of adenoviral proteins.¹⁹ Therefore, induction of autophagy by inactivating TOR may result in compromising the efficacious replication of the virus and the decrease in the oncolytic effect. Potential benefits of the combination of rapamycin and oncolytic viruses had been reported,³² but these are probably due to the inefficient inactivation of the AKT pathway by TORC1 inhibitors that results in increased activation of AKT.³³ However, dual TORC1 and TORC2 inhibitors are currently in clinical trials, and these experimental drugs should be incapable of activating autophagy without risking the unwanted inhibition of adenovirus protein synthesis. Our report places JNK as a key element in the regulation of autophagy and offers unexpected therapeutic opportunities. In this regard, tubulin inhibitors such as Taxol and vincristine induce the activation of JNK.³⁴ This type of chemotherapy can also induce degradation of members of the Bcl-2 family of proteins³⁵ and thus reinforce the pro-autophagy effect of JNK, and therefore may enhance the autophagic process without interfering with viral protein synthesis.

The role of JNK and autophagy in the immune response against cancer as well as the anti-pathogen innate immune response is still under examination^{36, 37}. Studies showing that autophagy plays a role in the innate immunity against viruses^{38, 39} currently coexist with reports indicating that viruses positively manipulate autophagy for replication and host cell lysis^{5, 40}. Therefore, it is important these aspects would be specifically examined in future clinical trials using oncolytic adenoviruses.

In summary, our mechanistic study of the regulation of autophagy in cancer cells treated with oncolytic adenovirus may have significance for the future design of clinical studies combining oncolytic adenoviruses and chemotherapy^{41, 42}.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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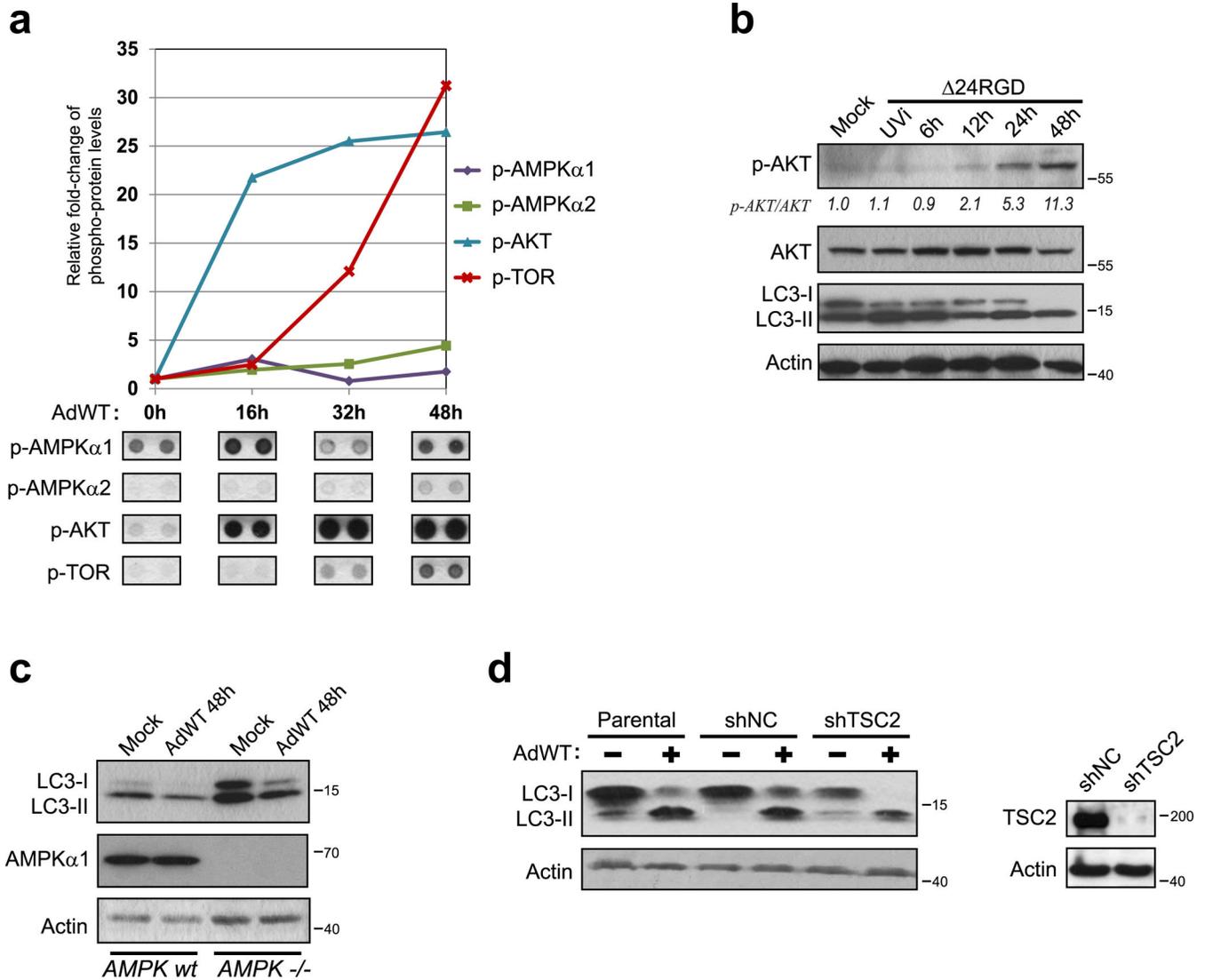


Figure 1. The canonical pathway of autophagy is not activated during adenovirus-mediated autophagy

(a) Kinetics of phosphorylated p-AMPK α 1 (T174), p-AMPK α 2 (T172), p-Akt (S473), and p-mTOR (S2448) protein levels in MCR5 lung fibroblasts infected with AdWT² at an MOI of 50. Whole-cell lysates were analyzed by using a human phospho-kinase antibody array (Supplementary Figure 1 and Table 1) at the indicated times after infection. The graph (top panel) represents the densitometry analyses of the duplicate dots (bottom panel) normalized against the controls. (b) U87 MG malignant glioma cells (ATCC) were infected with UV-inactivated or Delta-24-RGD (Δ 24RGD) adenovirus² (50 MOIs) for the indicated times and then cell lysates were analyzed by Western blotting for total and phospho-protein levels of Akt (S473). Protein levels of unprocessed LC3-I and the proteolytically cleaved LC3-II are shown as markers of autophagy. Actin is shown as a loading control. Quantification of the ratio p-Akt/Akt levels, normalized with the actin levels of expression, is indicated. UVi, UV-inactivated. (c) Wild-type *AMPK α 1* and *AMPK α 1*-null MEFs were infected with AdWT (100 MOIs) for 48 h and cell lysates were analyzed for expression of the indicated

proteins. **(d)** U251 MG cells (ATCC) were transfected with a pool of shNC or shTSC2 for 48 h and then infected with the Delta-24-RGD adenovirus (10 MOIs) for an additional 48 h. Whole-cell lysates were analyzed with use of anti-LC3 and anti-Actin antibodies (left panel). Right panel, Western blot illustrates the effect of shTSC2 and non-coding shRNA on TSC2 protein levels in U251 MG cells. All Western blots are representative of three independent experiments.

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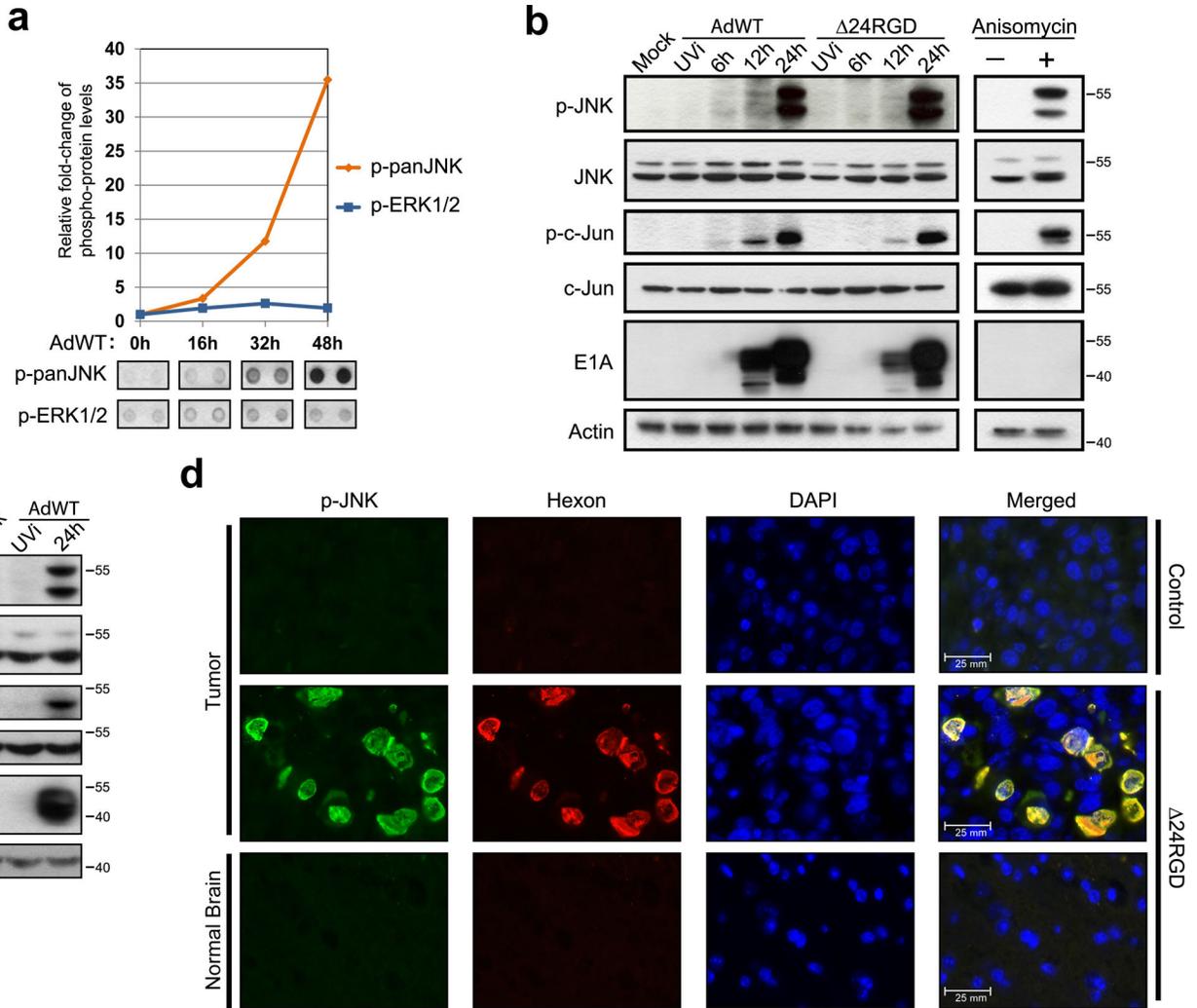


Figure 2. Adenoviral infection induces activation of the JNK pathway
(a) Kinetics of phosphorylated p-panJNK (T183/Y185, T221/Y223) and p-ERK1/2 (T202/Y204, T185/Y187) protein levels in immortalized MCR5 lung fibroblasts infected with AdWT (50 MOIs). Whole-cell lysates at the indicated times were analyzed by using a human phospho-kinase antibody array (Supplementary Figure 1). The graph (top panel) represents the densitometry analyses of the duplicate dots (bottom panel) normalized against the controls. **(b and c)** Whole-cell lysates from HeLa cervical cancer cells (b) (ATCC) or A549 lung cancer cells (c) infected with indicated adenoviruses at an MOI of 25 were analyzed for total and phosphorylated JNK and c-Jun protein levels. Anisomycin-treated cells (5 μ M, 30 min) were used as a positive control for JNK pathway activation. E1A expression is shown as evidence of adenoviral infection. Results represent at least three independent experiments. UVi, UV-inactivated. **(d)** Immunofluorescence analysis of brains from intracranial-bearing glioma mice treated with UV-inactivated or Delta-24-RGD adenoviruses. Anti-phospho-panJNK (green fluorescence) and anti-hexon (red fluorescence) antibodies were used to assess the co-localization (yellow) of p-JNK with viral proteins. DAPI was used for nuclear staining (blue). All fields in brain tissue from 4 mice per

treatment were scrutinized under deconvolution microscopy. The experiment was repeated twice with mounted tissue sections from each animal. Representative images for healthy brain (bottom panel) and tumor (middle panel) tissue from nude mice infected with Delta-24-RGD as well as images of tumor from UV-inactivated adenovirus-infected nude mice (top panel) are depicted. Scale bar = 25 μm (*see also* Fig. S2).

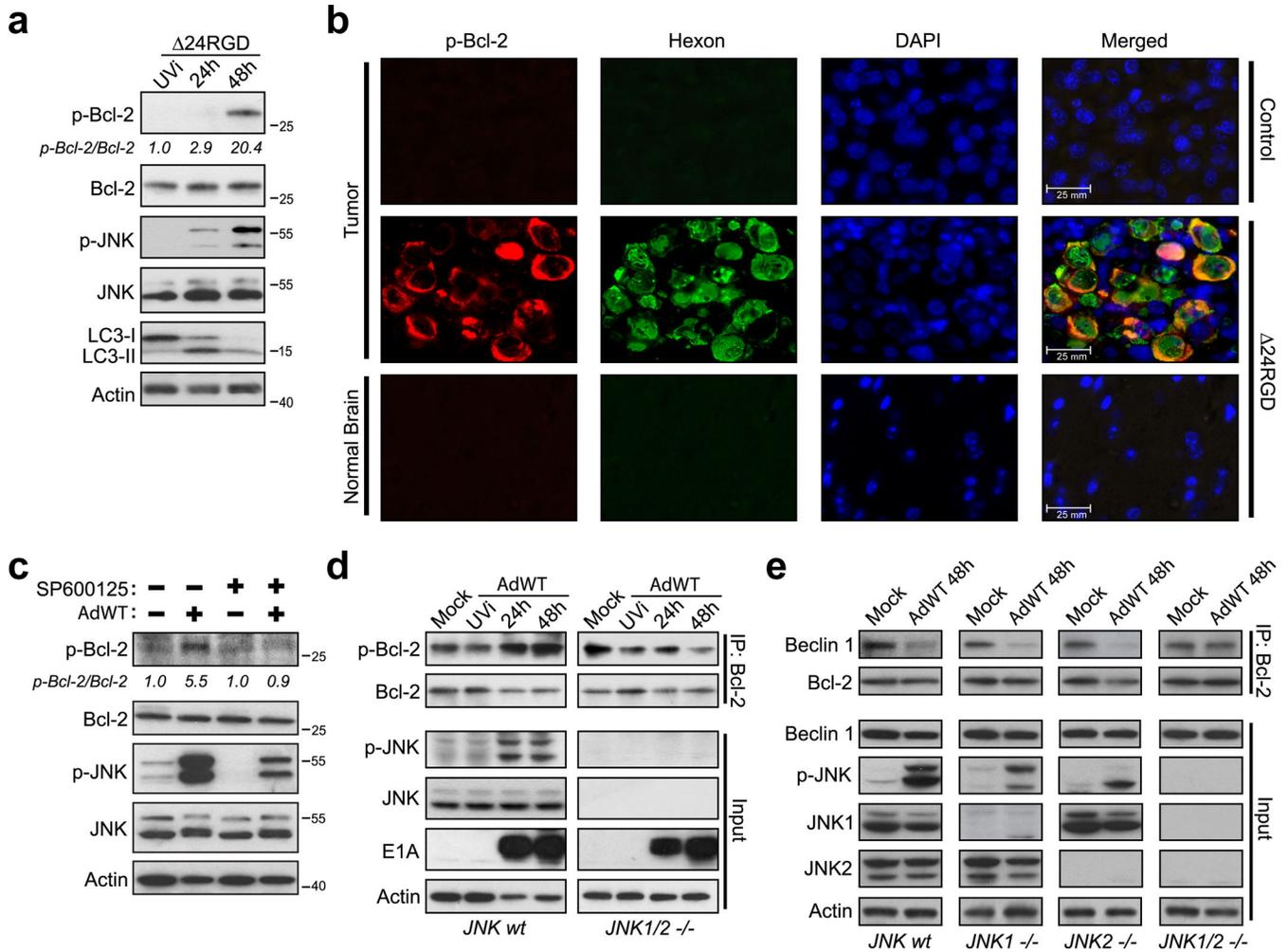


Figure 3. JNK1 and JNK2 mediate Bcl-2 phosphorylation and dissociation of the Bcl-2/Beclin 1 complex upon adenoviral infection

(a) Whole-cell lysates from U87 MG cells infected with UV-inactivated or Delta-24-RGD adenoviruses (50 MOIs) for 24 h or 48 h were analyzed for expression of the indicated proteins. Quantification of the ratio p-Bcl-2/Bcl-2 levels, normalized with the actin levels of expression, is indicated. (b) Immunofluorescence analysis of brains from intracranial-bearing glioma mice treated with UV-inactivated or Delta-24-RGD adenoviruses. Anti-phospho-Bcl-2 (red fluorescence) and anti-hexon (green fluorescence) antibodies were used to assess the co-localization of these two proteins (yellow). DAPI was used for nuclear staining (blue). All fields in brain tissue from 4 mice per treatment were examined under deconvolution microscopy. The experiment was repeated twice with mounted tissue sections from each animal. Representative images for healthy brain (bottom panel) and central tumor (middle panel) tissue from nude mice infected with Delta-24-RGD as well as images of central tumor tissue from UV-inactivated infected nude mice (top panel) are depicted. (c) Whole-cell lysates from A549 cells pretreated with DMSO or SP600125 (25 μM) 30 min before infection with AdWT (25 MOIs) for 48 h were analyzed for the expression levels of total and phospho-Bcl-2, and total and phospho-JNK. Actin is shown as a loading control. (d) Cell lysates from wild-type *JNK* (*JNK wt*) and *JNK1/2*^{-/-} MEFs mock-infected, or

infected with UV-inactivated or AdWT adenoviruses (100 MOIs) were immunoprecipitated with anti-Bcl-2 antibody and analyzed for total and phospho-Bcl-2 protein levels. Input samples (5%) for total and phospho-JNK, and E1A are shown. Actin was used as a loading control. (e) Cell lysates from *JNK wt*, *JNK1*^{-/-}, *JNK2*^{-/-}, or *JNK1/2*^{-/-} MEFs infected with AdWT (100 MOIs) for 48 h were immunoprecipitated with anti-Beclin1 antibody and analyzed for Bcl-2 with Beclin1 protein levels. Input sample (5%) was analyzed for p-JNK, JNK1, and JNK2 expression. Actin is shown as a loading control. All Western blots are representative of three independent experiments. UVi, UV-inactivated.

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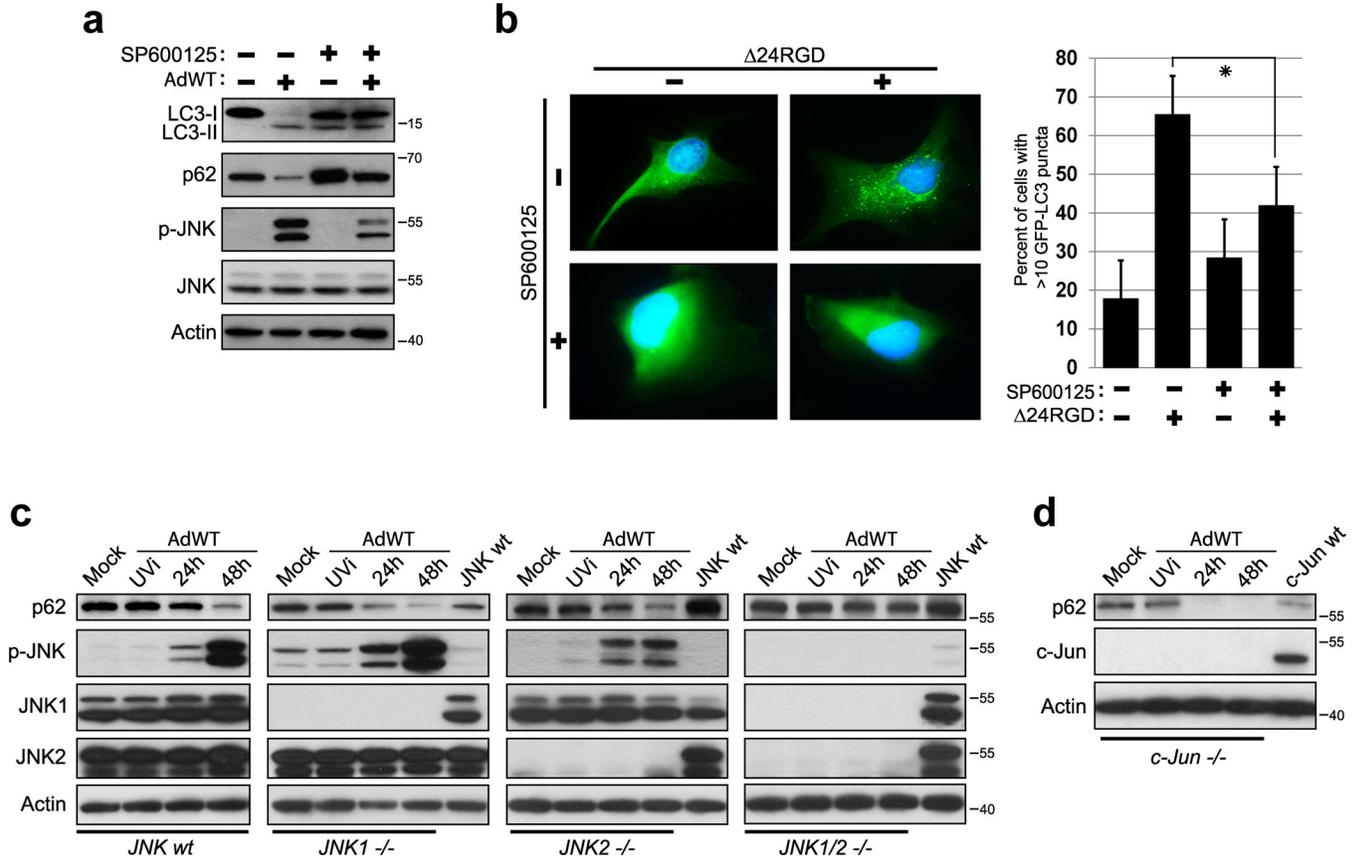


Figure 4. JNK1 and JNK2 may have redundant roles in the regulation of autophagy
(a) Whole-cell lysates from A549 cells pretreated with DMSO or SP600125 (25 μM) 30 min before infection with AdWT (25 MOIs) for 48 h were analyzed for expression levels of autophagy markers p62 and LC3-I to LC3-II conversion. Actin is shown as a loading control. **(b)** U87 MG cells transfected with an EGFP-LC3, as reported previously⁵ and treated with SP600125 or DMSO for 30 min before infection with Delta-24-RGD adenovirus (50 MOIs) for 48 h. Left panel, representative images of the GFP-LC3 puncta distribution visualized with use of deconvolution microscopy. Right panel, quantification of the percentage of cells presenting >10 GFP-LC3 puncta in 15 fields. * $P < 0.05$ (unpaired, two-tailed Student's *t*-test). **(c)** Whole-cell lysates from *JNK wt*, *JNK1 -/-*, *JNK2 -/-*, and *JNK1/2 -/-* MEFs infected with UV-inactivated or AdWT adenoviruses (100 MOIs) for 24 h or 48 h were analyzed for p62, p-JNK, JNK1, and JNK2 levels of expression. Actin is shown as loading control. **(d)** Whole-cell lysates from *c-Jun -/-* MEFs that were mock-infected, or infected with UV-inactivated or AdWT adenoviruses for 24 or 48 h (100 MOIs) were analyzed for expression of p62 and c-Jun. Actin is shown as loading control. UVi, UV-inactivated.

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