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Phosphorylation of Atg5 by the Gadd45 β -MEKK4-p38 pathway inhibits autophagy

Running title: Inhibition of autophagy by ATG5 phosphorylation

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Abstract

Autophagy is a lysosomal degradation pathway important for cellular homeostasis, mammalian development, cancer and immunity. Many molecular components of autophagy have been identified, but little is known about regulatory mechanisms controlling their effector functions. Here, we show that, in contrast to other p38 MAP kinase activators, the Gadd45 β -MEKK4 pathway specifically directs p38 to autophagosomes. This process results in an accumulation of autophagosomes through p38-mediated inhibition of lysosome fusion. Conversely, autophagic flux is increased in p38-deficient fibroblasts and Gadd45 β -deficient cells. We further identified the underlying mechanism and demonstrate that phosphorylation of the autophagy regulator Atg5 at threonine 75 through p38 is responsible for inhibition of starvation-induced autophagy. Thus, we show for the first time that Atg5 activity is controlled by phosphorylation and, moreover, that the spatial regulation of p38 by Gadd45 β /MEKK4 negatively regulates the autophagic process.

Keywords: Atg5, autophagy, Gadd45, p38, phosphorylation

Abbreviations: ASK1, apoptosis signaling kinase 1; Atg, autophagy-related; BMDM, bone marrow-derived macrophage; Gadd45 β , growth arrest and DNA damage 45 beta; GFP, green fluorescent protein; JNK, c-Jun N-terminal kinase; LC3, microtubule-associated protein 1 light chain 3; LPS, lipopolysaccharide; MAPK, mitogen activated protein kinase; M-CSF, macrophage colony-stimulating factor; MEF, mouse embryonic fibroblast; MEKK4, MAPK/ERK kinase kinase 4; MKK, MAP kinase kinase; PE, phosphatidylethanolamine; RFP, red fluorescent protein; TLR, toll-like receptor; WT, wildtype.

Introduction

Macroautophagy (hereafter referred to as autophagy) is a catabolic process, by which the cell degrades cytosolic content to supply metabolic processes with nutrients in order to maintain ATP production and macromolecular synthesis. Thus, autophagy acts as an efficient recycling mechanism in eukaryotic cells ¹. Cellular stress, e.g. nutrient deprivation, enhances autophagy as a survival mechanism during starvation. In addition, autophagy serves important functions in development, cancer, cell death and immunity in mammals ^{1,2}.

Autophagy is controlled by conserved key regulators known as autophagy-related (Atg) proteins ³. At the onset of the autophagy cascade, Atg6/Beclin-1 forms a complex with the class III phosphatidylinoside kinase Vps34, which induces expansion of the precursor membrane vesicle, the phagophore, via recruitment of additional Atg proteins. During expansion, the double membrane vesicle surrounds cytosolic content, and the completed vesicle, called autophagosome, finally fuses with lysosomes to degrade the autophagosomal content ³. Maturation of autophagosomes is regulated by two ubiquitin-like conjugation systems, namely the Atg8-phosphatidylethanolamine (PE) and the Atg5-12/16L1 conjugation system. The Atg5-Atg12 conjugate interacts with Atg16L1, which tethers the complex to phagophores and autophagosomes. This complex then acts as an E3-like ubiquitin ligase for LC3 lipidation. The conversion of LC3 to the PE-conjugated LC3-II form and its recruitment to the membrane serves as a well-accepted marker for autophagy.

Although Atg5-independent autophagy has been described ⁴, Atg5 is crucial for autophagy under most circumstances and Atg5-deficient mouse embryonic fibroblasts lack LC3 conversion and autophagy. Therefore, Atg5-deficient mice die postnatal due to their inability to cope with starvation during the neonatal period ⁵. In addition, Atg5 seems to be directly involved in the induction of apoptosis, as a calpain-generated fragment of Atg5 associates

with Bcl-x_L at mitochondria, resulting in the activation of the intrinsic apoptosis pathway ⁶. Thus, Atg5 could play a key role in controlling cell fate.

Although the function of Atg5 in constituting a complex with Atg12 and Atg16L1 is well determined, much less is known about the regulation of Atg5 activity during the autophagic process. Given the importance of autophagy in various biological processes, it is not surprising that it is tightly regulated by post-translational modifications of Atg proteins. For instance, acetylation of Atg5, Atg7, Atg8 and Atg12 by p300 inhibits autophagy ⁷, while TIP60 acetylates ULK1 inducing autophagy ⁸. In addition, the ULK1-Atg13-FIP200 autophagy-initiating complex is activated by direct phosphorylation of ULK1 by AMPK and inhibited by mTORC1 ⁹. Thus, acetylation and phosphorylation events might have opposing effects on the autophagic flux.

Gadd45 β is a member of a family comprising Gadd45 α , Gadd45 β /Myd118 and Gadd45 γ /CR6 ¹⁰. Gadd45 proteins are small acidic proteins without enzymatic activity that rather exert their function by protein-protein interactions. Best investigated is their regulatory role in mitogen-activated protein kinase (MAPK) signaling and ASK1, MEKK4 and MKK7 have been identified as Gadd45 interaction partners ^{11, 12}. Interestingly, while interaction of Gadd45 β with MKK7 inhibits JNK activation ¹¹, binding of Gadd45 β to MEKK4 activates the JNK and p38 pathway ¹². T cells from Gadd45 β -deficient mice show reduced activation of MAPKs, especially of p38, and impaired cytokine production upon T cell receptor triggering ¹³. In myeloid cells and hepatocytes, Gadd45 β has a survival function, which is thought to be mediated by inhibition of JNK ¹⁴⁻¹⁶.

Here, we demonstrate that p38 MAPK is selectively targeted to autophagosomes when activated by the Gadd45 β -MEKK4 signaling complex. At the autophagosomal membrane, active p38 phosphorylates Atg5 at threonine 75 leading to inhibition of autophagy. Thus, our

data show for the first time that Atg5 activity is directly controlled by p38-mediated phosphorylation in a Gadd45 β /MEKK4-dependent manner.

Results

Gadd45 β links p38 MAPK to autophagy

Gadd45 proteins have been described as regulators of MAPK signaling^{11, 12}. In NIH/3T3 fibroblasts coexpressing Gadd45 β and MEKK4 we detected activation of p38 MAPK but not of JNK or ERK (Figure 1a and Supplementary Figure 1). Expression of Gadd45 β alone resulted in detectable levels of phosphorylated p38, most likely due to endogenous MEKK4 (Figure 1a and data not shown). Gadd45 α - or Gadd45 γ expression induced only minor p38 phosphorylation, which was not increased by MEKK4 coexpression (Figure 1a). Since little is known about the spatial regulation of MAPK activation by Gadd45 proteins, we analyzed the subcellular localization of Gadd45-induced p38 activation. Thus, we prepared cytosolic and nuclear extracts of NIH/3T3 cells expressing Gadd45 β , MEKK4 or both. Surprisingly, most of the phosphorylated p38 was found in the cytoplasmic fraction when activated by Gadd45 β and MEKK4, in contrast to UV-irradiated cells, which showed nuclear localization of phosphorylated p38 (Figure 1b and Supplementary Figure 1e). Of note, coexpression of Gadd45 β with a dominant-negative MEKK4- Δ C construct lacking the kinase domain did not result in p38 activation (Figure 1b).

In order to verify the subcellular localization of Gadd45 β -mediated p38 activation we expressed Gadd45 β , MEKK4 or the combination of both in NIH/3T3 cells, stained for phosphorylated p38 and applied confocal microscopy. In agreement with previous results (Figure 1b and Ref. 12), MEKK4 localized cytosolically while Gadd45 β showed a nuclear and cytosolic localization when expressed without MEKK4. In MEKK4-Gadd45 β -coexpressing cells, Gadd45 β showed a prominent cytosolic staining and colocalization with MEKK4, consistent with these two proteins being interaction partners (Figure 1c and Supplementary Figure S1)¹². The expression of MEKK4 alone did not result in detectable

p38 activation (Figure 1c, upper row), which is in agreement with the kinase being in a closed conformation when Gadd45 proteins are absent¹⁷. Strikingly, phosphorylated p38 was detected in vesicular structures in the cytoplasm upon Gadd45 β expression (Figure 1c, middle panel). This unexpected localization of phosphorylated p38 was enhanced upon coexpression of MEKK4 and Gadd45 β (Figure 1c, lower panel), but was blocked by coexpression of Gadd45 β with dominant-negative MEKK4- Δ C (Supplementary Figure S1f).

To identify the nature of these dot-like structures, we co-expressed Gadd45 β and MEKK4 together with GFP-LC3, a widely accepted marker for autophagosomes¹⁸. Indeed, phosphorylated p38 co-localized with GFP-LC3 at cytoplasmic vesicles upon expression of Gadd45 β and MEKK4 (Figure 1d). In order to analyze whether activation of the Gadd45 β -MEKK4 pathway affects the number of GFP-LC3-positive vesicles, we transfected Gadd45 β , MEKK4 or both molecules into NIH/3T3 cells stably expressing GFP-LC3. 24 h post-transfection the number of GFP-LC3 positive dots was counted in 50 cells per condition. Expression of Gadd45 β alone showed a significantly higher number of GFP-LC3-positive puncta per cells compared to cells expressing GFP-LC3 alone ($p=0.0002$) or MEKK4 expression ($p=0.0149$, Figure 1e). Coexpression of Gadd45 β and MEKK4 increased the number of GFP-LC3-positive vesicles per cell even more ($p=0.001$ compared to Gadd45 β ; $p<0.0001$ compared to MEKK4). Based on these results, we conclude that the Gadd45 β -MEKK4-pathway directs activated p38 to autophagosomes, suggesting a role for p38 in autophagy regulation.

Gadd45 β in conjunction with MEKK4 but not ASK1 directs p38 to autophagosomes

We asked whether autophagy is influenced specifically by Gadd45 β and MEKK4. Thus, we investigated the potential role of other Gadd45 proteins and MAPK kinase kinases in p38 activation and autophagosome formation. Compared to other family members, Gadd45 β

induced a more pronounced punctuate localization of GFP-LC3, which co-localized with active p38, suggesting that Gadd45 β but not other Gadd45 members are associated with autophagy (Figure 2a). Moreover, ASK1, another Gadd45-interacting kinase¹¹, did not result in punctual localization of phosphorylated p38 (Figure 2b) or GFP-LC3 (Figure 2c). Thus, we conclude that Gadd45 β /MEKK4 but not Gadd45/ASK1 complexes direct p38 to autophagosomes.

Gadd45 β expression inhibits autophagic flux

Because autophagy is a dynamic process, the appearance of GFP-LC3-positive autophagosomes might be caused either by enhanced autophagy due to accelerated autophagosome formation or by the accumulation of autophagosomes resulting from reduced fusion with lysosomes¹⁹. To analyze the autophagic flux, we transfected Gadd45 β , MEKK4 or both together with a tandem reporter construct. This allows identification of autophagosomes (RFP⁺GFP⁺) and autolysosomes (RFP⁺GFP⁻), since GFP fluorescence is lost upon lysosomal acidification while RFP fluorescence remains stable²⁰. While LC3 puncta formation was increased upon starvation in MEKK4 single transfected cells, starvation had no effect on puncta formation in Gadd45 β only and Gadd45 β /MEKK4 transfected cells (Figure 3a). Importantly, double transfected cells showed increased autophagosome numbers but no change in autolysosomes upon starvation, suggesting inhibition of autophagic flux upon Gadd45 β and MEKK4 expression (Figure 3b). As an independent measure of autophagic flux we analyzed degradation of GFP-LC3 by immunoblotting, since LC3 is rapidly degraded but GFP is quite resistant to lysosomal hydrolysis¹⁹. In line with the microscopy data, cleavage of GFP-LC3 was impaired in GFP-LC3-expressing NIH/3T3 cells when Gadd45 β was expressed with or without MEKK4, while MEKK4 alone had no effect compared to the control cells (Figure 3c and 3d).

Active p38 inhibits autophagy

Next, we asked whether or not the kinase activity of p38 was instrumental to the inhibitory effect on autolysosome formation. To address this question, we expressed Gadd45 β and MEKK4 in NIH/3T3 cells in the absence or presence of the p38 inhibitor SB203580 and analyzed the localization of active p38. In control samples the expected vesicular localization of active p38 was detected, whereas inhibition of p38 activity by SB203580 resulted in a homogenous distribution of phosphorylated p38 in the cytoplasm as well as in the nucleus (Figure 4a). Therefore, kinase activity is required to target p38 to autophagosomes.

To investigate the impact of p38 kinase activity on autophagy in more detail, we used the GFP-LC3 expressing NIH/3T3 cells and cultured them in HBSS to induce autophagy. Cells were grown in the absence or presence of SB203580 to inhibit p38 and autophagosome formation was monitored by live cell imaging. Quantification of GFP-LC3 puncta per cell at different time points revealed that the number of autophagosomes was about doubled when p38 kinase activity was inhibited (Figure 4b).

Next, we used p38 α knock-out MEFs as a model system lacking p38 kinase activity²¹. p38 α -deficient MEFs and their wildtype (WT) counterparts were transfected with GFP-LC3. 24 h post-transfection, cells were starved in HBSS and GFP-LC3 puncta were analyzed by confocal microscopy. Upon starvation, p38 α -deficient MEFs showed a higher number of GFP-LC3 puncta than control WT MEFs (Figure 4c). Thus, the number of GFP-LC3-positive puncta increased with both activation and inhibition of p38 (compare Figure 1c and 1e vs. 4c). This at first glance contradictory result prompted us to investigate the autophagic flux in p38 α -deficient cells. To this end, we starved WT and p38 α -deficient MEFs for up to 16 h and analyzed the conversion of endogenous LC3-I to the PE-conjugated LC3-II form by immunoblotting. Cells were cultured in the presence of bafilomycin A1 for the last 2 h in

order to prevent lysosomal degradation of LC3-II. In the absence of bafilomycin A1, LC3-I and LC3-II were hardly detectable in these MEFs (data not shown). p38 α -deficient MEFs showed an earlier accumulation of LC3-II and in higher numbers compared to WT MEFs (Figure 4d). Thus, autophagic flux was strongly promoted in the absence of p38.

p38 phosphorylates Atg5

So far we established that active p38 was recruited to autophagosomes and its kinase activity was necessary for the inhibition of autophagosome-lysosome fusion. Therefore, we reasoned that p38 phosphorylates a target protein on autophagosomal vesicles and that this phosphorylation event influenced autophagic activity. In search for a potential kinase substrate, we analyzed the sequences of those Atg proteins that are essential components of autophagosomes. The objective was to find conserved S/T-P motifs recognized by MAPKs²². We identified two potential phosphorylation sites in Atg5 at T75 and T249 (Figure 5a). The TP motif at position 75/76 is conserved in all species analyzed ranging from yeast to men, while the TP motif at position 249/250 is not present in *Saccharomyces cerevisiae*, *Drosophila melanogaster* and *Rattus norvegicus*. To test whether p38 directly phosphorylates Atg5, we purified recombinant Atg5 and incubated it with active p38 and radioactive ATP. Caspase-3 and GST were used as negative controls, while ATF2, a known p38 target, served as positive control. Although ATF2 phosphorylation was stronger, phosphorylation of Atg5 by p38 could readily be detected (Figure 5b). To determine whether p38 phosphorylates Atg5 at T75 or T249, recombinant Atg5 proteins with alanine substitutions were analyzed by in vitro kinase assays. The p38 kinase phosphorylated the T249A mutant as efficiently as WT Atg5, whereas the T75A mutant was not phosphorylated by p38, indicating that p38 phosphorylates Atg5 at T75 (Figure 5c).

To show that p38-mediated phosphorylation of Atg5 is important for autophagy regulation in living cells, we took advantage of Atg5-deficient MEFs⁵, which were reconstituted either with WT or the phosphorylation-defective T75A mutant of Atg5. These MEFs were transfected with the RFP-GFP-LC3 tandem reporter and with MEKK4 together with either Gadd45 β or an empty vector control. 24 h post-transfection, cells were starved for 4 h and analyzed by confocal microscopy. While WT Atg5 expressing cells showed an accumulation of large RFP⁺GFP⁺ autophagosomes and decreased RFP⁺GFP⁻ autolysosomes upon Gadd45 β expression, the appearance of autophagosomes and autolysosomes was not affected by Gadd45 β expression in Atg5 T75A expressing MEFs (Figure 5d). Thus, we conclude that the effect of the Gadd45 β -MEKK4-p38 pathway on autophagy is mediated by phosphorylation of Atg5 at T75.

Phosphorylation of Atg5 at Thr75 inhibits autophagy

In order to investigate functional consequences of Atg5 phosphorylation, we used reconstituted Atg5-deficient MEFs as above and additionally introduced a T75E mutant, which mimics constitutive phosphorylation. First, we analyzed autophagic flux by immunoblotting for endogenous LC3. Cells were either starved in HBSS or cultured in complete medium. Moreover, starvation was performed in the absence or presence of E64 and pepstatin A to inhibit lysosomal proteases and prevent degradation of LC3-II. In agreement with previous studies⁵, Atg5-deficient MEFs showed no conversion of LC3-I to the lipidated LC3-II form (Figure 6a). As expected, WT MEFs accumulated LC3-II upon starvation when lysosomal proteases were inhibited by E64 and pepstatin A, consistent with enhanced autophagic flux. WT Atg5-reconstituted MEFs showed a similar LC3 pattern as MEFs from WT mice (Figure 6b). Of note, the levels of the Atg5-Atg12 complex were similar in the reconstituted MEFs when compared to WT MEFs (Figure 6a and 6b).

Importantly, Atg5 T75A mutant cells showed higher amounts of LC3-II when starved in the presence of protease inhibitors, indicating a higher autophagic activity (Figure 6b). In contrast, less LC3-II was detectable in MEFs reconstituted with the T75E mutant. Similar results were obtained when autophagy was monitored by acridine orange, a lysosomotropic fluorescent dye, and flow cytometry (Supplementary Figure S2). Taken together, these data strongly suggest that p38-mediated phosphorylation of Atg5 at T75 inhibits autophagy.

Next, we analyzed autophagosome formation by electron microscopy. WT but not Atg5-deficient cells showed typical double-membrane structures, i.e. autophagosomes, upon starvation (Figure 6c). MEFs expressing the T75A mutants contained autophagosomes even when cultured in complete medium, suggesting enhanced basal autophagy (Figure 6c). In contrast, T75E-expressing MEFs showed no autophagosome formation, even under starvation conditions (Figure 6c). Furthermore, reconstituted MEFs were transfected with a RFP-GFP-LC3 reporter and starved for 4 h or left in complete culture medium. RFP-GFP-LC3 was mostly diffusely distributed in cells expressing WT or the T75E mutant Atg5, which were cultured in complete medium, with only a few RFP⁺GFP⁺ vesicles (Figure 6d). As expected, RFP⁺GFP⁺ LC3-puncta were increased upon starvation in cells expressing WT Atg5, but not in T75E mutant cells (Figure 6d and 6e). In both genotypes, we detected only few RFP⁺GFP⁻ dots, i.e. autolysosomes. Importantly, T75A-expressing MEFs had highly increased numbers of LC3-puncta, especially of RFP⁺GFP⁻ autolysosomes, suggesting that preventing phosphorylation of Atg5 strongly promotes autophagic flux (Figure 6d and 6e).

Increased autophagy in Gadd45 β -deficient cells upon LPS stimulation

Finally, we asked whether Gadd45 β -mediated regulation of autophagy plays a physiological role. Since Gadd45 β expression is induced by LPS²³ and autophagy is initiated upon TLR activation²⁴, we investigated whether autophagy was altered in Gadd45 β -deficient cells upon

LPS treatment. Therefore, we activated WT and Gadd45 β -deficient MEFs with LPS in the absence and presence of bafilomycin A1. Without inhibition of lysosome acidification, the ratio of LC3-I to LC3-II was comparable between Gadd45 β -deficient and WT MEFs, although total LC3 expression was increased in the knock-out cells (Figure 7a). This higher LC3 expression in Gadd45 β -deficient MEFs could indicate a general increase in autophagy in these cells, since expression of autophagy genes is induced during ongoing autophagy¹⁹. Indeed, blocking lysosomal degradation by bafilomycin A1 showed a LPS- and time-dependent accumulation of LC3-II, which was much more pronounced in Gadd45 β -deficient compared to WT MEFs (Figure 7a). Quantification of LC3 abundance over three independent experiments substantiated the observed increase in autophagic flux upon LPS stimulation in the absence of Gadd45 β (Figure 7b).

In order to substantiate our findings, we analyzed the role of Gadd45 β on autophagy in primary cells. To this end, bone marrow-derived macrophages (BMDMs) were generated from WT and Gadd45 β -deficient mice, stimulated with LPS and analyzed by immunoblotting. Similar to MEFs, a LPS- and time-dependent accumulation of LC3-II was observed in Gadd45 β -deficient BMDMs in the presence of bafilomycin A1 (Figure 8a). In contrast, WT BMDMs did not show increased LC3-II. Therefore, we conclude that Gadd45 β controls the amplitude of TLR-mediated autophagy.

Discussion

Although the pathways mediating autophagy have been elucidated in recent years, regulatory mechanisms modulating this process are poorly characterized. Most previous work focused on two kinase complexes, the class III PI3K complex and mTORC1, which are positive and negative regulators of autophagy, respectively ³. Both kinase complexes act in the very early phase of autophagy induction, but very little is known about how autophagy is regulated at later stages. Here we show that phosphorylation of Atg5 blocks autophagosome maturation downstream of initiation processes. We furthermore identified p38 as the regulator that mediates phosphorylation of Atg5 and identified threonine-75 as its substrate amino acid. Importantly, threonine-75 is evolutionary conserved from yeast to men indicating the relevance of this posttranslational modification. Interestingly, activation of the Gadd45 β -MEKK4 pathway specifically and not other p38 stimuli such as ASK1 (another Gadd45 β -interacting kinase) or UV irradiation induced p38 translocation to autophagosomes. In summary, we provide a detailed molecular mechanism implicating p38 in autophagy regulation downstream of mTOR and class III PI3K complexes by phosphorylation of Atg5.

At first glance it is surprising that autophagosome numbers increase upon p38 activation, e.g. by expression of Gadd45 β and MEKK4, as well as upon p38 inactivation, i.e. by pharmacological inhibition or gene knock-out. How can these results be explained? An increase in autophagosome numbers can be detected either because fusion of autophagosomes with lysosomes is blocked, or because of a general increase in autophagy, which can lead to increased expression of Atg genes ¹⁹. Thus, the mere number of autophagosomes does not allow conclusions about enhanced or repressed autophagy. In addition, one has to keep in mind that next to stimulation-induced (e.g. starvation-induced) autophagy every cell has a certain level of basal autophagy ²⁵. We show here that the

expression of Gadd45 β leads to p38-mediated phosphorylation of Atg5, which blocks autolysosome formation. In this situation, more autophagosomes can be detected as basal autophagy is inhibited. In contrast, in the p38-deficient situation, we detected more autophagosomes upon starvation, i.e. stimulation-induced autophagy. In this setting, autophagic flux is enhanced as Atg5 cannot be phosphorylated by p38 and, thus, fusion of autophagosomes with lysosomes proceeds.

What could be the physiological role for autophagy regulation by Gadd45 β and MEKK4? Gadd45 β is induced upon different cellular stresses, inflammatory cytokines and LPS^{10,23}. As LPS signals via TLR4, which was shown to induce autophagy²⁴, we investigated whether Gadd45 β affects LPS-induced autophagy. Indeed, we show that autophagy was increased in the absence of Gadd45 β suggesting that induction of Gadd45 β by the LPS-TLR4 pathway acts as a negative feedback loop to dampen TLR-induced autophagy.

MAPKs, especially JNK1, have been implicated in autophagy regulation before³. JNK1 phosphorylates Bcl-2 at serine and threonine residues in a N-terminal regulatory loop, releasing Beclin1 from Bcl-2 and initiating autophagy^{26,27}. Thus, JNK1 affects an early step of autophagy while the Gadd45 β -MEKK4-p38 pathway described here acts further downstream. Furthermore, the role of p38 in the regulation of autophagy is controversial, since both promotion and inhibition of autophagy by p38 have been reported²⁸⁻³¹. However, none of these studies provided insights into the underlying molecular mechanisms.

Recently, p38-interacting protein (p38IP) was reported to interact with Atg9 and to be required for starvation-induced trafficking of Atg9³². Active p38 sequesters p38IP away from Atg9 and, thus, indirectly impairs Atg9 trafficking and autophagosome formation³². Unfortunately, the enzymatic activity of p38 was not addressed, suggesting that not kinase activity but competition between p38 and Atg9 for p38IP is crucial in this context. We show

here an additional and direct role for p38 in autophagy regulation, involving its enzymatic activity. p38-mediated phosphorylation of Atg5 inhibits autophagy upstream of the fusion of autophagosomes with lysosomes. Importantly, the Atg5 conjugation system, which is also an essential component of the classical autophagy machinery, operates independently from Atg9³³. Another distinction between our study and the one performed by Webber and Tooze is the p38-activating stimulus. We show that p38 is specifically directed to autophagosomes upon activation of the Gadd45 β -MEKK4 pathway. The Atg9-p38IP-interaction was inhibited by UV or anisomycin treatment, both of which activate p38 and JNK via MLK7, a MAPK kinase kinase different from MEKK4³⁴, suggesting that these two regulatory pathways are activated by different stimuli. In conclusion, our work provides first insights into a novel role of Gadd45 β in autophagy, showing for the first time a selective and spatial regulation of Atg5 activity through Gadd45 β -MEKK4-mediated p38 activation, which exerts a negative regulatory role for autophagosome maturation.

Materials and methods

Antibodies and reagents

The antibodies used for immunoblotting and immunoprecipitation were: Atg5 (AP1812a, Abgent), Atg5 (D1G9), caspase-3, ERK, LC3 (G40), phospho-ERK, JNK, phospho-JNK, p38, phospho-p38, PARP (all Cell Signaling), LC3 (2G6; Nanotools, Teningen, Germany), MEKK4 (Sigma), Flag (M2; Sigma), HA (12CA5, Roche), c-MYC (9E10; Santa Cruz Biotechnology), V5 (Invitrogen) and tubulin (Sigma). HRP-conjugated goat anti-rabbit IgG was obtained from Santa Cruz Biotechnology. HRP-conjugated goat anti-rat IgG, donkey anti-goat IgG, goat anti-mouse IgG1, IgG2a and IgG2b were acquired from SouthernBiotech. Bafilomycin A1, E64, LPS and pepstatin A were purchased from Sigma. LysoTracker was obtained from Invitrogen.

Cell culture

NIH/3T3 murine fibroblasts were cultured as described previously³⁵. NIH/3T3 cells stably expressing GFP-LC3 or RFP-GFP-LC3 were generated by retroviral transduction. MEFs deficient of p38 α and their WT counterparts were kindly provided by Dr. Angel Nebreda³⁶. Atg5-deficient and respective control MEFs were a kind gift of Dr. Noboru Mizushima⁵. Gadd45 β -deficient MEFs and their WT counterparts were generated according to standard protocols and immortalized by transfection of a plasmid encoding the SV40 large T antigen. Gadd45 β -deficient mice have been described before¹⁴. All MEFs were cultured in Dulbecco's Modified Eagle Medium (DMEM high glucose, Invitrogen) supplemented with 10% fetal calf serum (PAA) and 50 μ g/mL of each penicillin and streptomycin (Invitrogen). Cells were transfected with Lipofectamine 2000 (Invitrogen), Dreamfect (OZ Biogene), JetPei (Peqlab) or Nanofectin (PAA) according to manufacturers' instructions. MEFs stably expressing Atg5 WT and mutants were selected using 1 mg/ml G418. Bone marrow-derived

macrophages (BMDMs) were differentiated out of bone marrow from femurs and tibias of either WT or Gadd45 β -deficient mice, 8 to 10 weeks of age, according to published protocols³⁷. Briefly, bone marrow cells were cultured for 7 days in DMEM containing 10% fetal bovine serum (Sigma), 2 mM glutamine, 50 μ g/mL of each penicillin and streptomycin and 5% M-CSF-containing supernatant from 3T3 cells, with feeding on day 1, 3 and 5. On day 7, cells were detached from the surface with trypsin, put into new M-CSF-free medium and subsequently used for stimulation experiments. Successful differentiation was validated by F4/80 staining and flow cytometry.

Plasmids, recombinant protein expression and site directed mutagenesis

Expression constructs for Gadd45 proteins³⁸, MEKK4³⁹, Atg5⁶, Ask1⁴⁰, GFP-LC3¹⁸ and RFP-GFP-LC3²⁰ have been described previously. A dominant-negative mutant of MEKK4 lacking the C-terminal kinase domain (MEKK4- Δ C) was cloned into pEF4-myc/his (Invitrogen) using the following primers: forward 5'-GGG GTA CCA TGA GAG ACG CCA TCG CC-3'; reverse 5'-GCT CTA GAC TCC ACT GGA CGG TCG TCC-3'. Expression and purification of recombinant Atg5 was done as reported before⁶. Atg5 mutants were generated by using the QuickChange site-directed mutagenesis kit (Stratagene) and verified by DNA sequencing. Primer sequences are available upon request. Atg5 WT and mutants were cloned into either pET15b (Novagen) for recombinant protein expression or into pEF-myc-cyto (Invitrogen) for eukaryotic cell transfection.

Fluorescence microscopy

4x10⁴ NIH/3T3 cells grown on a coverslip in a 12-well dish were transiently transfected with 4 μ g plasmid DNA using Optifect or JetPei transfection reagent. 24 h later, cells were washed with PBS and fixed with 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min. The cells were washed again and permeabilized with 0.05% saponin and 4% BSA in PBS for 30 min.

Thereafter, cells were incubated with the appropriate antibody over night in blocking solution (4% BSA, 0.05% saponin in PBS) at 4°C. Coverslips were washed several times with PBS and incubated with Alexa-Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. For nuclear staining, the cells were washed several times and incubated with 4',6'-diamidino-2-phenylindole dihydrochloride (DAPI, 100 ng/ml) for 10 min. Coverslips were then mounted in fluorescence mounting medium (DakoCytomation) and analyzed in confocal laser scanning microscopes (LS2 and LS5, Leica Microsystems, and LSM780, Zeiss) or on a spinning disc microscope (Perkin Elmer).

Transmission electron microscopy

For electron microscopy, MEFs were plated at a density of 2×10^6 cells in 175 cm² cell culture flasks. After 24 h the cells were starved in Hank's buffered salt solution (HBSS) minimal medium or cultivated in supplemented DMEM (Invitrogen) and harvested 24 h later. Subsequently, cultured cells were harvested, washed in PBS, centrifuged for 5 min at 200 g, and fixed in 2% glutaraldehyde/0.1 M sodium cacodylate buffer (pH 7.4) at room temperature for 1 h. Cells were washed twice in PBS and postfixed in 2% OsO₄/PBS at room temperature for 1 h, dehydrated in ethanol, and embedded in Epon (Serva). 50 nm thin sections were contrasted with uranylacetate and lead citrate and examined on a transmission electron microscope (Zeiss EM 902A, Zeiss).

Immunoblotting and immunoprecipitations

Immunoblotting was performed as described previously³⁵. For co-immunoprecipitation analysis 2×10^6 NIH/3T3 cells were transiently transfected as described above. Cells were lysed in lysis buffer (20 mM Tris/HCl, pH 7.4, 1% Triton X-100, 10% glycerol, 150 mM NaCl, 1 mM PMSF and 1 µg/ml each of leupeptin, aprotinin, chymostatin and pepstatin A) for 15 min on ice and centrifuged (15 min, 20,000 g). Subsequently, the immunoprecipitation

was performed with 2 μg antibody coupled to protein G beads (Sigma) for 4 h at 4°C. Finally, beads were washed three times with 750 ml ice-cold lysis buffer and analyzed by immunoblotting.

In vitro kinase assay

The radioactive kinase assay cocktail contained 250 $\mu\text{Ci/mL}$ [γ - ^{32}P]ATP, 10 mM MgCl_2 , 5 mM MnCl_2 , 25 mM β -glycerophosphate, 25 mM HEPES pH 7.5, 5 mM benzamidine, 0.5 mM dithiothreitol and 1 mM Na_2VO_3 . 200 ng of recombinant active p38 (ProQinase/Biomol) was incubated with 1 μg of recombinant substrate proteins for 1 h at 37°C while shaking. The reactions were stopped by adding 5x SDS-sample buffer (155 mM Tris-HCl pH 6.8, 5% SDS, 50% glycerol, 25% β -mercaptoethanol) and denaturing at 96°C for 5 min. The samples were analyzed by 12% SDS-PAGE. The gel was transferred onto PVDF membrane, followed by autoradiography.

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Conflict of interest

The authors declare no conflict of interest.

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Titles and legends to figures

Figure 1. Gadd45 β specifically activates p38 MAPK through MEKK4 and recruits p38 to autophagosomes. (a) NIH/3T3 cells were transfected with either V5-tagged Gadd45 α or Gadd45 β or Gadd45 γ , HA-tagged MEKK4 or combinations thereof. As a negative control untransfected cells were included. 24 h post-transfection, cellular lysates were prepared and analyzed by immunoblotting using the indicated antibodies. (b) NIH/3T3 cells were transfected with either V5-tagged Gadd45 β and HA-tagged MEKK4 or both. As negative and positive controls untransfected and UV-irradiated cells were included. As an additional control, a C-terminal deleted MEKK4 (MEKK4- Δ C) construct lacking the kinase domain was transfected together with Gadd45 β . MEKK4- Δ C retains the ability to interact with Gadd45 β but lacks enzymatic activity. 24 h post-transfection, cytosolic and nuclear extracts were prepared and analyzed by immunoblotting using the indicated antibodies. Caspase-3 and PARP were used as markers for cytosolic and nuclear compartments, respectively. (c) NIH/3T3 cells were transiently transfected with either V5-tagged Gadd45 β and HA-tagged MEKK4 alone or with both constructs. 24 h post transfection, the cells were fixed and stained with anti-HA (red), anti-V5 antibody (red), anti-phospho-p38 (green) and DAPI (blue). In the lower panel, MEKK4 is not visible since not more than three molecules could be stained due to technical reasons. Nevertheless, cytoplasmic staining of Gadd45 β in the absence of any nuclear signal indicates co-expression of MEKK4. Subsequently, samples were analyzed by confocal microscopy. Bars, 10 μ m. (d) NIH/3T3 cells were transfected with V5-tagged Gadd45 β , HA-tagged MEKK4 and GFP-LC3. The cells were stained with anti-V5 (blue), LC3-GFP (green) and anti-phospho-p38 (red) and analyzed as described in (c). Boxed areas are enlarged below. Bars, 10 μ m. (e) NIH/3T3 cells stably expressing GFP-LC3 were transfected with the indicated constructs. 24 h later wide-field

pictures were taken and the number of GFP-LC3-positive autophagosomes per cell was counted in 50 cells per condition. For statistics two-tailed Student's t-test was performed (* $P=0.0149$, ** $P=0.001$, *** $P<0.0001$).

Figure 2. Localization of p38 in dot-like structures is specific for the interaction between Gadd45-proteins and MEKK4. (a) NIH/3T3 cells were transiently transfected with either V5-tagged Gadd45 α , V5-tagged Gadd45 β or V5-tagged Gadd45 γ together with GFP-tagged LC3 and HA-tagged MEKK4. 24 h post-transfection, the cells were stained with anti-V5 (blue), LC3-GFP (green) and anti-phospho-p38 (red). Subsequently, samples were analyzed by confocal microscopy. Bars, 10 μm . (b) NIH/3T3 cells were transiently transfected with V5-tagged Gadd45 β and HA-tagged ASK1. 24 h after transfection the staining was performed with anti-V5 (green), anti-HA (red) and endogenous phosphorylated p38 (blue). Subsequently, samples were analyzed by confocal microscopy. Bars, 10 μm . (c) NIH/3T3 cells were transfected with V5-tagged Gadd45 β , HA-tagged ASK1 and GFP-LC3. After 18 h, cells were fixed and stained with anti-HA (red) and anti-V5 antibody (blue). Bars, 10 μm .

Figure 3. Gadd45 β inhibits starvation-induced autophagic flux. (a) NIH/3T3 cells stably expressing RFP-GFP-LC3 were transiently transfected with V5-tagged Gadd45 β and HA-tagged MEKK4. 24 h post-transfection, the cells were fixed and stained with anti-HA or anti-V5 (AF633). Subsequently, samples were analyzed by confocal microscopy. Bars, 10 μm . (b) Quantification of the experiments shown in (a). The number of autophagosomes (RFP $^+$ GFP $^+$) and autolysosomes (RFP $^+$ GFP $^-$) per cell was counted in 50 cells per condition. (c) NIH/3T3 cells stably expressing GFP-LC3 were transiently transfected as in (a). 24 h post-transfection, the cells were either starved in HBSS for 4 h or kept in full medium. Subsequently,

processing of GFP-LC3 was analyzed by immunoblotting. **(d)** Densitometric quantification of the GFP to GFP-LC3 ratio of results shown in (b).

Figure 4. Lack of active p38 accelerates autophagy. **(a)** NIH/3T3 cells were transiently transfected with V5-tagged Gadd45 β and HA-tagged MEKK4. 24 h post-transfection, the cells were fixed and stained for endogenous phosphorylated p38 (blue), HA-tagged MEKK4 (red) and the V5 tagged Gadd45 β (green). Additionally, the cells were treated with 500 nM of the p38 inhibitor SB203580 or DMSO as solvent control. Bars, 10 μ m. **(b)** GFP-LC3 expressing NIH/3T3 cells were cultured in the presence or absence of 500 nM SB203580 in HBSS and followed by live cell imaging for the indicated time points. Autophagosomes were counted in 40 cells and the mean number of autophagosomes per cell was calculated from two independent visual fields. Error bars represent s.e.m. **(c)** Wildtype and p38^{-/-} MEFs were transfected with GFP-LC3 as a marker for autophagy. 24 h post-transfection cells were cultured for 4 h in HBSS medium to induce autophagy. Subsequently, samples were fixed and analyzed by confocal microscopy (left panel). Bars, 10 μ m. The number of autophagosomes was counted in 40 cells per condition (right panel). **(d)** WT and p38^{-/-} MEFs were cultured for up to 16 h in HBSS medium to induce autophagy. In order to prevent LC3-II degradation, cells were pretreated with 100 nM bafilomycin A1 for 1 h. Subsequently, cellular lysates were prepared and analyzed by immunoblotting using the indicated antibodies. Densitometric quantification of the LC3-II to ERK ratio is depicted at the bottom.

Figure 5. Atg5 is a substrate for p38. **(a)** The protein sequences of Atg5 proteins from different species were analyzed for potential phosphorylation sites of p38 (XS/TPX consensus). Two putative phosphorylation sites could be identified at threonine 75 and threonine 249 of murine Atg5. **(b)** The indicated proteins were tested for phosphorylation by

p38 in an in vitro kinase assay. His-Caspase-3 and GST served as a negative control, whereas GST-ATF2 was used as a positive control. (c) In vitro kinase assay with WT His-Atg5 as well as with His-Atg5, in which T75 and T249 had been mutated to alanine, respectively. GST served as a negative control. (d) Atg5^{-/-} MEF cells stably reconstituted with WT or a T75A mutant of Atg5 were transfected with MEKK4 and an RFP-GFP-LC3 reporter either with or without Gadd45 β co-expression. 24 h post-transfection, cells were starved for 4 h in HBSS. Subsequently, cells were fixed, stained for Gadd45 β expression and analyzed by confocal microscopy for RFP-GFP-LC3 localization. Bars, 10 μ m.

Figure 6. Atg5 phosphorylation by p38 inhibits the process of autophagy. (a) WT and Atg5^{-/-} MEF were cultured for 4 h in HBSS medium to induce autophagy or left in full culture medium. E64 and pepstatin A was used to prevent lysosomal degradation of LC3-II. Subsequently, cellular lysates were prepared and analyzed by immunoblotting using anti-LC3 and anti-Atg5 antibodies. Tubulin served as a loading control. (b) MEFs reconstituted with WT, T75A or T75E mutated Atg5 were treated and analyzed as described in (a). Densitometric quantification of the LC3-II to tubulin ratio is depicted below the loading control. (c) WT, Atg5-deficient (Atg5^{-/-}) and Atg5^{-/-} MEF cells stably reconstituted with T75A or T75E mutants of Atg5 were cultured for 4 h in HBSS to induce autophagy. Control cells were left in full culture medium. Subsequently, samples were fixed and analyzed by transmission electron microscopy. (d) Atg5-deficient MEF cells reconstituted with WT Atg5, Atg5-T75A or Atg5-T75E were transfected with RFP-GFP-LC3. 24 h post-transfection, cells were either starved in HBSS for 4 h or left in full culture medium. Subsequently, cells were fixed and analyzed by confocal microscopy for RFP-GFP-LC3 localization. Bars, 10 μ m. (e) Quantification of the results shown in (d). Numbers of autophagosomes and autolysosomes were counted in 50 cells per condition.

Figure 7. Gadd45 β suppresses autophagy upon LPS stimulation in MEFs. (a) MEFs from WT and Gadd45 β -deficient mice were stimulated in vitro with 100 ng/ml LPS for the indicated time periods. In order to prevent LC3-II degradation, half of the cells were treated with 100 nM bafilomycin A1 for the last 2 h of culturing. Subsequently, cellular lysates were prepared and analyzed by immunoblotting for expression of phospho-p38, total p38, Atg5 and LC3. Tubulin served as a loading control. Results shown are representative for three independent experiments. (b) Quantification of the results shown in (a). The intensities of LC3-II bands were normalized to the tubulin loading control. Data are represented as mean of three independent experiments \pm s.e.m.

Figure 8. Gadd45 β deficiency enhances LPS-induced autophagy in bone marrow-derived macrophages. (a) Bone-marrow-derived macrophages (BMDMs) from WT and Gadd45 β -deficient mice were stimulated in vitro with 100 ng/ml LPS for the indicated time periods. In order to prevent LC3-II degradation, half of the cells were treated with 100 nM bafilomycin A1 for the last 2 h of culturing. Subsequently, cellular lysates were prepared and analyzed by immunoblotting for expression of phospho-p38, total p38, Atg5 and LC3. β -actin served as a loading control. Results shown are representative for two independent experiments. (b) Schematic representation of the role of the Gadd45 β -MEKK4-p38 pathway on Atg5 and autophagic flux. See discussion for details.