Published in final edited form as:
*Oncogene*. 2011 April 7; 30(14): 1727–1732. doi:10.1038/onc.2010.553.

# IKK-Dependent, NF-κB-Independent Control of Autophagic Gene Expression

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## Abstract

The induction of mammalian autophagy, a cellular catabolic bulk-degradation process conserved from humans to yeast, was recently shown to require IKK, the upstream regulator of the NF-κB pathway. Interestingly, it was shown that this response did not involve classic NF-κB. Thus, the mechanism by which IKK promotes stimulus-induced autophagy is largely unknown. Here we investigate the role of IKK/NF-κB in response to nutrient deprivation, the classic autophagy-inducing stimulus. IKK and both the classic and non-canonical pathways of NF-κB are robustly induced in response to cellular starvation. Notably, cells lacking either catalytic subunit of IKK (IKKα or IKKβ) fail to induce autophagy in response to cellular starvation. Importantly, we show that IKK activity but not NF-κB, controls basal expression of the pro-autophagic gene LC3. We further demonstrate that starvation induces the expression of LC3 and two other essential autophagic genes, ATG5 and Beclin-1, in an IKK-dependent manner. These results demonstrate that the IKK complex is a central mediator of starvation-induced autophagy in mammalian cells and suggest that this requirement occurs at least in part through the regulation of autophagic gene expression. Interestingly, NF-κB subunits are dispensable for both basal and starvation-induced expression of pro-autophagic genes. However, starvation-induced activation of NF-κB is not inconsequential as increases in expression of anti-apoptotic NF-κB target genes such as cIAP2 is observed in response to cellular starvation. Thus, IKK likely plays multiple roles in response to starvation by regulating NF-κB-dependent anti-apoptotic gene expression as well as controlling expression of autophagic genes through a yet undetermined mechanism.

### Keywords

Autophagy; IKK; NF-κB; LC3; ATG5

## Introduction

The IKK/NF-κB signaling axis is a major molecular regulator of inflammatory signaling and stress responses in mammalian cells. NF-κB is a transcription factor composed of homo- and hetero-dimeric complexes of five subunits (p65/RelA, c-Rel, RelB, p105/p50, and p100/p52), which regulate the expression of a numerous target genes in response to a variety of cellular signals and stresses (Gilmore, 2006). NF-κB is known be activated by two different

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pathways each requiring a distinct IKK subunit. The classical pathway involves IKKβ, regulates NF-κB complexes containing p65 and c-Rel, and is most well understood in response to inflammatory cytokines like TNFα. The alternative or non-canonical pathway is activated primarily in lymphoid tissue, is IKKα-dependent, and controls NF- κB complexes containing RelB and p52 (Hayden and Ghosh, 2004). Regulation of NF-κB activity is quite diverse and occurs in response to various signals including DNA damage, cell cycle, and metabolic conditions like glucose availability or redox status (Barré and Perkins, 2007; Bednarski *et al.*, 2009; Bubici *et al.*, 2006). Therefore there is much interest in understanding the role IKK/NF-κB activity plays in facilitating a cell's response to its changing environment.

IKK is a large multi-subunit complex consisting of a regulatory subunit (NEMO) and two catalytic subunits (IKKβ and IKKα). IKK activates NF-κB by phosphorylating a class of inhibitory molecules called Inhibitor of κB (IκB), marking them for proteosome-dependent degradation. IKK-dependent phosphorylation of IκB results in NF-κB nuclear translocation and transcriptional activation of target genes (Hacker and Karin, 2006). To date, investigation of IKK activity has focused on its role as the critical mediator of NF-κB regulation. More recently IKK, but not NF-κB, was demonstrated to be required for induction of the cellular catabolic process macro-autophagy (herein referred to as autophagy) (Criollo *et al.*, 2009), however, the mechanism by which IKK controls autophagy is unclear.

Autophagy is a bulk-degradation process utilized by cells to maintain energy homeostasis and adapt to nutrient availability. Autophagy is characterized by double-membraned organelles called autophagosomes, which engulf cytoplasmic components including macromolecules, organelles, and damaged cellular material. Autophagosomes fuse with the lysosome where its contents are degraded and recycled by digestive enzymes (Klionsky, 2004). In so doing, autophagy serves as a housekeeping mechanism, preventing accumulation of potentially toxic molecules. Autophagy is also induced in response to changing metabolic states, the most classically studied model being cellular starvation. During starvation cells up-regulate autophagy, promoting turnover of its own contents as a means supplying essential nutrients (Klionsky, 2004).

The machinery that controls autophagy is conserved from yeast to humans, but regulation of autophagy in higher organisms is more complex. Signaling pathways important for regulation of autophagy have also been implicated in human disease progression, thus there is major interest in understanding how these pathways contribute to autophagy and how autophagy promotes disease pathogenesis (Levine and Kroemer, 2008). Here we investigate the role IKK/NF-κB signaling pathways play in regulating autophagy in response to the classic autophagy-inducing stimulus cell starvation. Data presented herein demonstrate that both classical and non-canonical pathways of NF-κB activation are induced in response to cellular starvation. Importantly, both catalytic subunits of the IKK complex (IKKα and IKKβ) are required for starvation-induced autophagy. IKK subunits are further demonstrated to control expression of pro-autophagic genes, but this effect is independent of NF-κB activity. These results suggest that IKK-dependent induction of autophagy occurs in part through regulation of the genes necessary for this process.

## Results and Discussion

### Cellular starvation induces both classical and non-canonical NF-κB pathways

To understand the role IKK/NF-κB signaling plays in regulation of autophagy, the activity of these molecules was analyzed in cells undergoing nutrient starvation, the classic autophagy-inducing stimulus. Treatment of wildtype (WT) mouse embryonic fibroblasts

(mEFs) with starvation media resulted in phosphorylation and degradation of IκBα and phosphorylation of NF-κB subunit p65, markers associated with activation of the classical NF-κB pathway (Figure 1A). An important step during induction of autophagy occurs when LC3, an essential component of the autophagosome machinery, is cleaved, lipidated, and incorporated into an elongating autophagosome. This processed form of LC3 (LC3-II) migrates faster on a gel than its precursor (LC3-I) and therefore the ratio of these species is often used as an indicator of increased autophagy (Klionsky *et al.*, 2008). Notably, activation of NF-κB occurred rapidly following starvation and preceded LC3 processing (Figure 1A). Nuclear extracts prepared from nutrient deprived mEFs displayed increased binding to a consensus κB DNA oligonucleotide in an electrophoretic mobility shift assay (EMSA) (Figure 1B). Importantly, nutrient-deprivation induced DNA binding occurred in a cyclical manner, consistent with most examples of stimulus-induced NF-κB activation (Renner and Schmitz, 2009; Saccani *et al.*, 2001), but signal intensity was less than that observed with TNFα stimulation. EMSA supershift analysis with antibodies against NF-κB subunits demonstrated that this complex consists of classical NF-κB hetero-dimer p65/p50 (Figure 1B). Finally, target gene expression was assessed in order to determine if starvation induces activation of functional NF-κB. Expression of the classic NF-κB target gene NFKBIA was dramatically increased in response to starvation (Figure 1C). Specifically, a four-fold increase in NFKBIA expression occurred within the first two hours of starvation and a secondary increase in expression (almost 10-fold) was observed following 8 hours of starvation. Taken together, nutrient starvation results in increased signaling markers of activated NF-κB, DNA binding, and target gene expression, indicating that activation of canonical NF-κB occurs in response to cellular starvation.

Processing of p100/p52 was monitored in order to determine if the non-canonical NF-κB pathway is also activated in response to starvation. Whereas activation of classical NF-κB activity occurred rapidly following starvation, p100/p52 processing was observed under periods of prolonged starvation, where accumulation of p52 was observed at 12 hours (Figure 1D). Total levels of unprocessed p100 also increased in response to starvation suggesting that prolonged nutrient stress may promote increased NF-κB activity through regulation of subunit expression. In this regard, p100/p52 gene NFKB2 is itself an NF-κB target gene (Lombardi *et al.*, 1995) raising the possibility that NF-κB participates in a feed-forward mechanism to achieve maximal activity through control of subunit expression. The contribution of increases in p100 expression versus induced p100 processing is currently unclear; nevertheless starvation results in marked accumulation of p52. More investigation will be necessary to determine a role for non-canonical NF-κB signaling in response to starvation.

### IKK controls basal and starvation-induced expression of pro-autophagic genes

Having observed activation of IKK and multiple NF-κB subunits in response to cellular starvation, the role of IKK in regulating autophagy was next examined. Cells lacking IKK subunits fail to induce LC3 processing in response to starvation as monitored by the conversion of the unprocessed LC3-I species to the cleaved, lipidated, autophagy-specific species LC3-II (Figure 2A). Measurements using alternative autophagy detection techniques including GFP-LC3 puncta, and endogenous LC3 subcellular localization, confirm the results of Criollo *et al.* (2009) that IKK is required for starvation-induced autophagy (data not shown). This data indicates that IKK catalytic subunits are important for autophagy, which may imply distinct activities for IKKα and IKKβ. Pharmacological inhibition of IKK in WT mEFs with multiple IKK-specific compounds confirmed that IKK is required for starvation-induced autophagy (data not shown). Importantly, cells lacking IKKα or IKKβ displayed a marked decrease in LC3 protein expression compared to WT mEFs (Figure 2A).

Having observed IKK-dependent effects on LC3 expression and noting that starvation induces IKK activity and NF-κB target gene expression, the expression of pro-autophagic genes in response to starvation was measured. Surprisingly, expression of LC3, BECN1, and ATG5 mRNA was induced following 12 hours of starvation (Figure S1). Starvation-induced expression changes were consistently statistically significant increases compared to untreated controls. Slight variability in expression increases was observed between experiments (eg. Compare WT cells in Figure 2B to 3B) but this variability is likely the result of culture conditions as we observed an inverse correlation between passage number or confluency of the cells and their ability to activate starvation-induced pro-autophagic gene expression. Considering that IKK subunits are required for starvation-induced autophagy and that starvation induces robust increases in both NF-κB target genes and genes required for autophagy, IKK-dependent expression of LC3, BECN1, and ATG5 was measured in cells under basal growth and in response to starvation. Real Time PCR analysis of LC3 mRNA levels in WT, IKKα-/-, and IKKβ-/- mEFs grown in basal medium confirmed that IKK deficient cells have decreased LC3 mRNA levels, which correlates with protein expression (Figure 2B). Moreover, IKK was required for starvation-induced expression LC3, Beclin-1, and Atg5 mRNA (Figure 2B). WT mEFs but not IKK deficient cells grown in starvation media for 12 hours display two- to five- fold increases in expression of these genes. As a control, the classic NF-κB target gene NFKBIA is induced in WT and IKKα-/- mEFs but not cells lacking IKKβ (basal levels are increased compared to WT, but fail to induce in response to starvation), consistent with a role for IKKβ in controlling activation of the classical NF-κB pathway (Figure 2B).

In order to confirm that starvation-induced changes in autophagic gene expression is IKK-dependent, WT mEFs were subjected to 12 hours of starvation in the presence or absence of the cell permeable NEMO-Binding Domain (NBD) peptide which disrupts an interaction between IKK catalytic and regulatory subunits (May *et al.*, 2000). Consistent with genetic loss of IKK subunits, pharmacological inhibition of IKK with the NBD peptide blocked starvation-induced expression of LC3, BECN1, and ATG5 (Figure 2C). Similar results were observed when WT cells were treated with the IKKβ-specific small molecule inhibitor Compound A (data not shown) (Ziegelbauer *et al.*, 2005). These results demonstrate that starvation-induced pro-autophagic gene expression requires IKK catalytic activity.

## IKK-dependent expression of pro-autophagic genes does not require NF-κB

Previous studies have implicated NF-κB in both positive and negative regulation of autophagy. For example, expression of p65 is sufficient to induce autophagy and is required for Beclin-1 expression in T-cells (Copetti *et al.*, 2009). On the other hand, p65 blocks TNFα-stimulated autophagy in Ewing sarcoma cells (Djavaheri-Mergny *et al.*, 2006). Given these different results, a requirement for NF-κB subunits in regulation of autophagic gene expression was explored. p65-/- mEFs were grown in basal and starvation media for 18 hours and gene expression was measured with Real Time PCR. MEFs lacking p65 displayed equivalent basal expression levels of LC3, BECN1 and ATG5. Moreover, p65 deficient mEFs consistently displayed a statistically significant increase in the expression of these genes in response to starvation (Figure 3A). Starvation-induced expression levels in p65 null cells were slightly lower than those for observed in WT cells which may be the result of slight variations between mEF cell lines or may indicate a slight contribution from p65 in promoting the expression of these genes (see below). Importantly, p65 null cells fail to induce NFKBIA expression in response to starvation; over an 18 hour starvation timecourse expression levels in p65-/- cells increase only two-fold compared to nearly 10-fold increases observed in WT mEFs (Figures S2 and 1C). These results indicate that p65 is required for starvation-induced expression of classic NF-κB target genes but not for starvation-induced pro-autophagic gene expression (see below as well).

In some instances NF-κB family member c-Rel compensates for classical NF-κB activation in p65 deficient cells (Hoffmann *et al.*, 2003). In order to determine if c-Rel activity was responsible for autophagic gene expression in p65-deficient cells we assayed p65/c-Rel double knock out cells (p65/c-Rel DKO), which should be devoid of any classical NF-κB pathway activation, for basal and starvation-induced expression of LC3, BECN1, and ATG5. p65/c-Rel DKO cells displayed intact autophagic gene expression confirming that the classical NF-κB pathway is dispensable for LC3, BECN1, and ATG5 expression (Figure 3A). Given that p65/c-Rel DKO mEFs displayed expression levels equivalent to WT control cells we believe that the slight decreases in expression of these genes observed in p65-/- cells is likely not specific for p65 activity. Finally, a requirement for non-canonical NF-κB subunits in basal and starvation-induced autophagic gene expression was investigated using mEFs lacking either RelB or p100/p52. Cells lacking these subunits demonstrated that non-canonical NF-κB pathway is dispensable for autophagic gene expression as mutant and WT mEFs displayed equivalent levels of LC3, BECN1 and ATG5 under both basal and starved conditions (Figure 3B). Notably, RelB and p52 deficient cells also display normal starvation-induced expression levels of NFKBIA indicating that this gene is indeed regulated by classical NF-κB dimers in response to starvation. While starvation-induced expression of pro-autophagic genes does not require non-canonical NF-κB activity, future studies should explore the significance of starvation-induced changes in non-canonical NF-κB target gene expression. These studies should investigate starvation timepoints later than 12 hours since starvation-induced processing of p100 to p52 does not occur until this point (Figure 1D). Together, these data suggest that genetic loss of NF-κB has no effect on either basal or starvation-induced autophagic gene expression.

The results presented in this report support the findings of Criollo et al (2009) that the IKK complex is essential for the induction of starvation-induced autophagy. We extend these findings by showing that both IKK subunits (IKKα and IKKβ) control basal and starvation-induced expression of a subset of genes required for autophagy. Given that loss of either IKK catalytic subunit is sufficient to block both starvation-induced autophagy and autophagic gene expression it will be important to determine if IKK subunits participate in a co-dependent mechanism or if they have distinct functions that converge on the similar phenotypes. Importantly, pro-autophagic gene expression is not controlled by IKK-dependent activation of NF-κB subunits, even though activation of both classical and non-canonical NF-κB pathways is observed in response to starvation. Starvation-induced NF-κB activity is likely not without consequence however, as we find increased expression of anti-apoptotic and pro-survival NF-κB target genes like cIAP2, Bnip3, and Bcl-xL in response to starvation (data not shown). NF-κB-dependent expression of these genes could have important implications for balancing survival (autophagy) and death (apoptosis), a field that continues to remain under intense scrutiny (Levine and Yuan, 2005). Starvation-induced IKK activity therefore has multiple functions, first in controlling autophagy through regulation of autophagic gene expression and secondly by controlling an independent pathway leading to activation of NF-κB. It is likely that there are other key functions of IKK in regulating autophagy.

The role transcription factors play in regulating autophagy through gene expression changes is poorly understood. A recent report demonstrated that Skp2 expression, which inhibits autophagy, is controlled by non-canonical NF-κB complexes in response to DNA damage (Barré and Perkins, 2010). The authors showed that NF-κB plays an intricate role in coordinating autophagic and apoptotic pathways in response to various DNA damaging agents, which also required p53 activity. In addition, p53-dependent transcription of DRAM or TIGAR can directly activate or inhibit autophagy respectively, and cytoplasmic p53 inhibits autophagy by a transcription-independent mechanism (Bensaad *et al.*, 2009; Crighton *et al.*, 2006; Tasdemir *et al.*, 2008). E2F transcription factors influence autophagy

through direct regulation of pro-autophagic genes DRAM, LC3 and ATG1, and by indirect regulation of ATG5 (Polager *et al.*, 2008). Interestingly, an emerging theme in the literature suggests that IKK/NF-κB and E2F family transcription factors are involved in complex crosstalk. IKK and NF-κB can regulate cell cycle by inhibiting E2F target gene transcription (Araki *et al.*, 2008). Furthermore, basal and hypoxia induced expression of the pro-autophagic gene BNIP3 is controlled through NF-κB-dependent antagonism of E2F-1 in cardiomyocytes (Shaw *et al.*, 2008). Further investigation is necessary to determine how IKK/NF-κB and E2F signaling pathways participate in cross-regulatory mechanisms to influence autophagy.

In summary, we propose that IKK plays two roles in promoting survival in response to starvation. Firstly, IKK is required for a signaling-dependent mechanism that promotes autophagosome formation (Criollo *et al.*, 2009 and Figure 2A). IKK-dependent control of LC3 expression supports this role by ensuring sufficient gene products for the machinery required for autophagosome formation. Secondly, under periods of extended starvation (12 hours), IKK activity is required to up-regulate autophagic components, likely to replace gene products that have been diminished by prolonged autophagy. Surprisingly, the requirement for IKK subunits in regulating gene expression does not occur through modulation of NF-κB activity. Future studies should therefore identify IKK-dependent substrates important for the cell's response to nutrient deprivation in order to better understand how IKK controls mammalian autophagy.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

We are grateful to Dr. Alexander Hoffmann for the p65/c-Rel DKO cells and to Dr. Denis Guttridge for the RelB-/- and p52-/- cells. We thank members of the Baldwin lab for thoughtful discussion and manuscript feedback. Research support provided by NIH grants CA75080, AI035098 and the Waxman Cancer Research Foundation.

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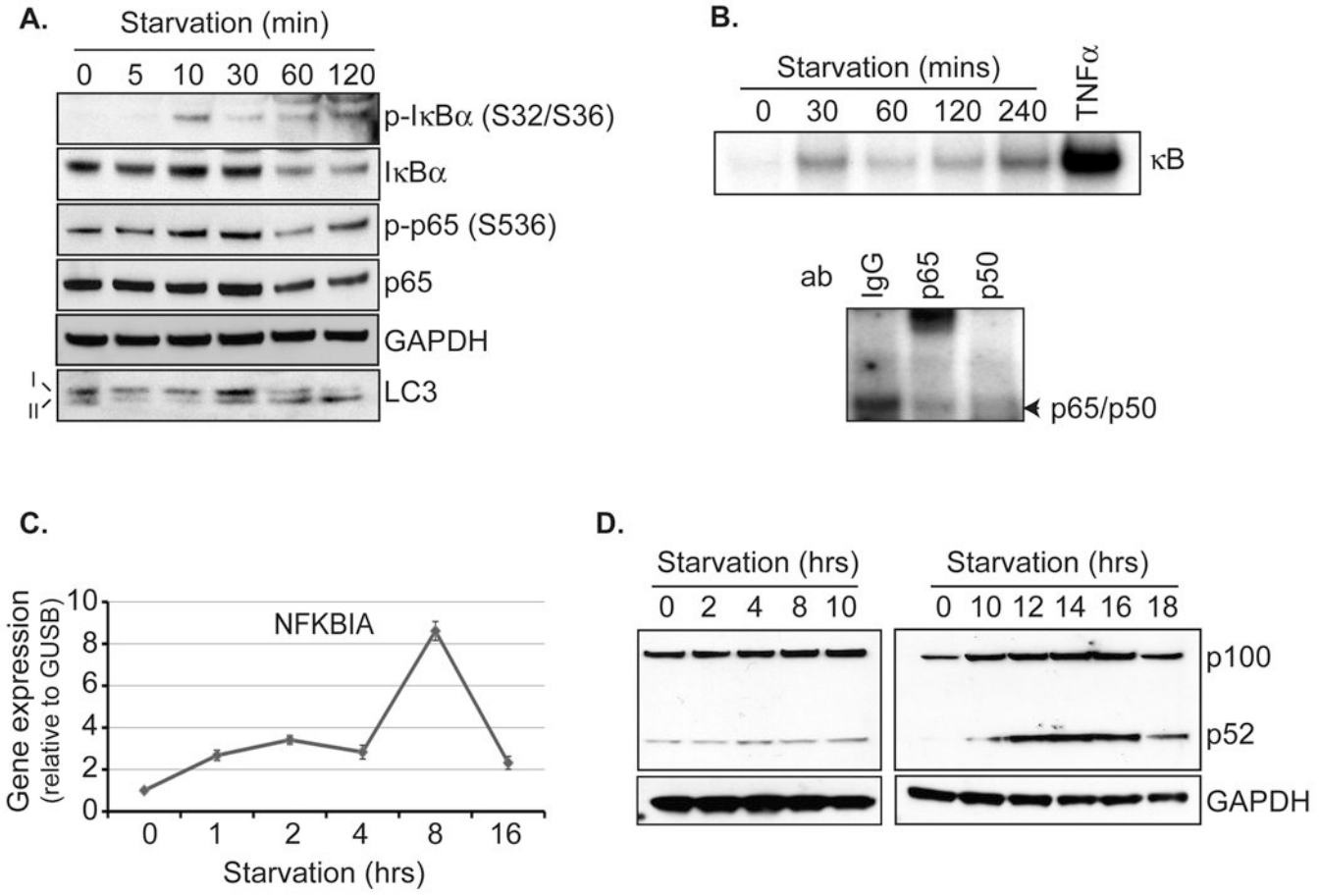


**Figure 1. IKK/NF-κB signaling is activated by nutrient stress**

(**A**) Wildtype mouse embryonic fibroblasts (WT mEFs) were starved in Hanks' Balanced Salt Solution (HBSS, Sigma: 55021C) for a two-hour timecourse and cells were harvested at the indicated time points for whole cell lysates (WCL). WCL were subjected to westernblot analysis for phosphorylated IκBα (phospho, Cell Signaling Technology (CST) 9246; total, CST: 4812), phosphorylated p65 (phospho, CST: 3033; total CST: 4764), and LC3 processing (CST: 3868). (**B**) Nuclear extracts were prepared for WT mEFs treated with a starvation timecourse for the indicated time points. NF-κB DNA binding was assessed by EMSA using a consensus κB oligonucleotide (Promega: E3292) (upper panel). Nuclear extracts from WT mEFs starved for 240 minutes were used for supershift anlysis of NF-κB complexes using antibodies against p65 and p50 (CST: 3034; Santa Cruz: 7178, respectively) (**C**) WT mEFs were starved for a 16-hour time course and total RNA was collected from cells at the indicated timepoints. Real Time PCR was used to assess the levels of the classic NF-κB target gene NFKBIA (Applied Biosystems Taqman gene expression assays, ABI: Mm00477798_m1). (**D**) WCLs were prepared from WT mEFs treated for starvation time course and activation of non-canonical NF-κB was interrogated by processing of p100 to p52 by westernblot analysis (CST: 4882).

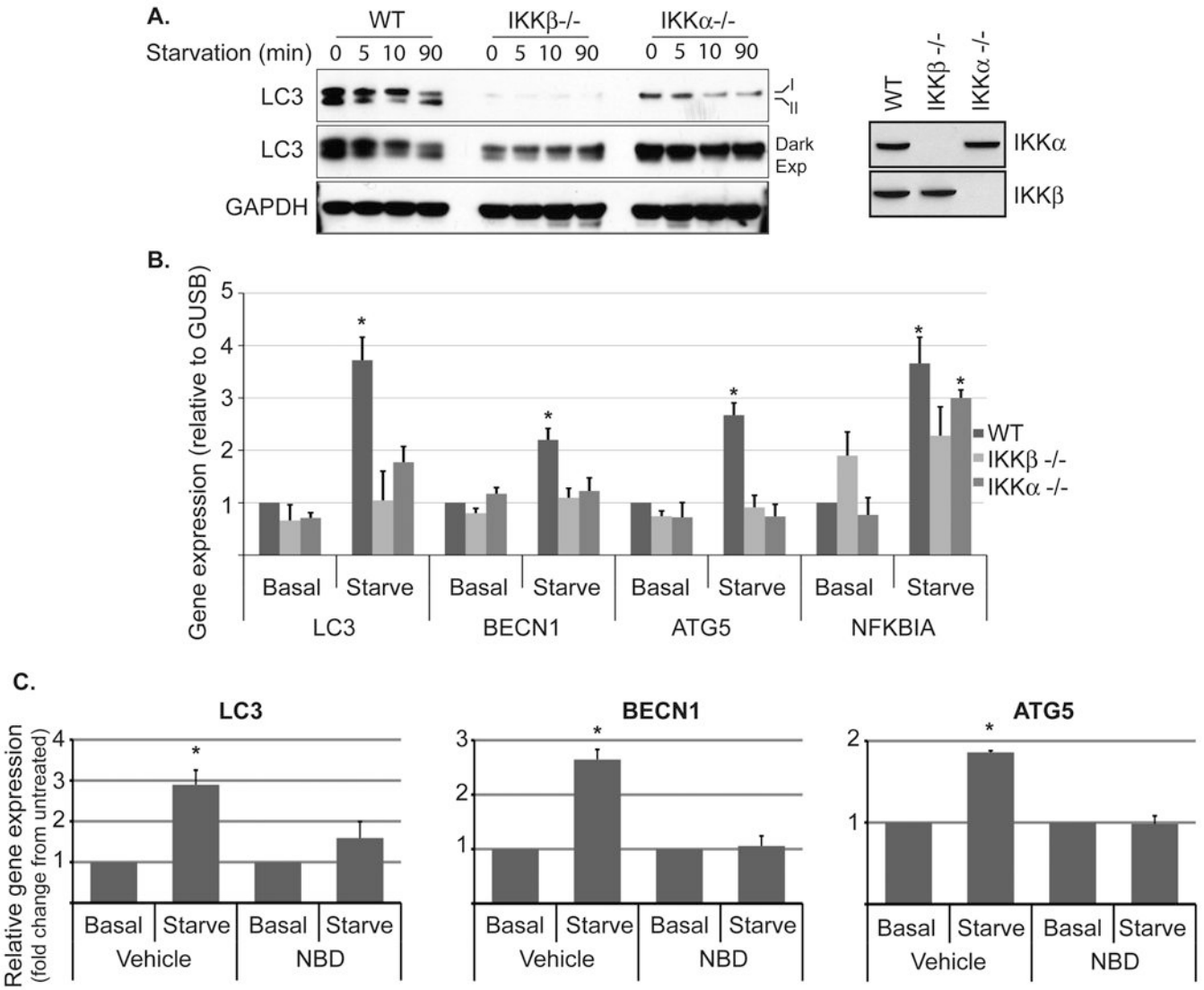


**Figure 2. IKK is required for basal and starvation-induced expression of pro-autophagic genes**

(**A**) WT or IKK deficient mEFs (IKKβ -/-, IKKα-/-) were grown in basal or starvation media (HBSS) for 90 minutes and WCLs were prepared. Induction of autophagy was measured by LC3 processing. (**B**) WT, IKKβ -/-, and IKKα-/- cells were grown in basal or starvation media for 12 hours total RNA was collected for cDNA synthesis. Expression of LC3, ATG5, BECN1, and NFKBIA were measured using Taqman gene expression assays (ABI: LC3, Mm00458724_m1; BECN1, Mm01265461_m1; ATG5, Mm00504340_m1). Samples were normalized to GUSB expression (ABI: GUSB, Mm03003537_m1). Statistically significant differences were measured by Student's *t-test* (*<.05) (**C**) WT mEFs were treated with vehicle control or IKK inhibitor NEMO Binding Domain (NBD) peptide (100μM) 1 hour prior to starvation. Cells were then starved in the presence NBD peptide or control for 12 hours. Gene expression was measured by Real Time PCR analysis. Data is represented as fold-change over expression of cells grown in basal media (*<.05).

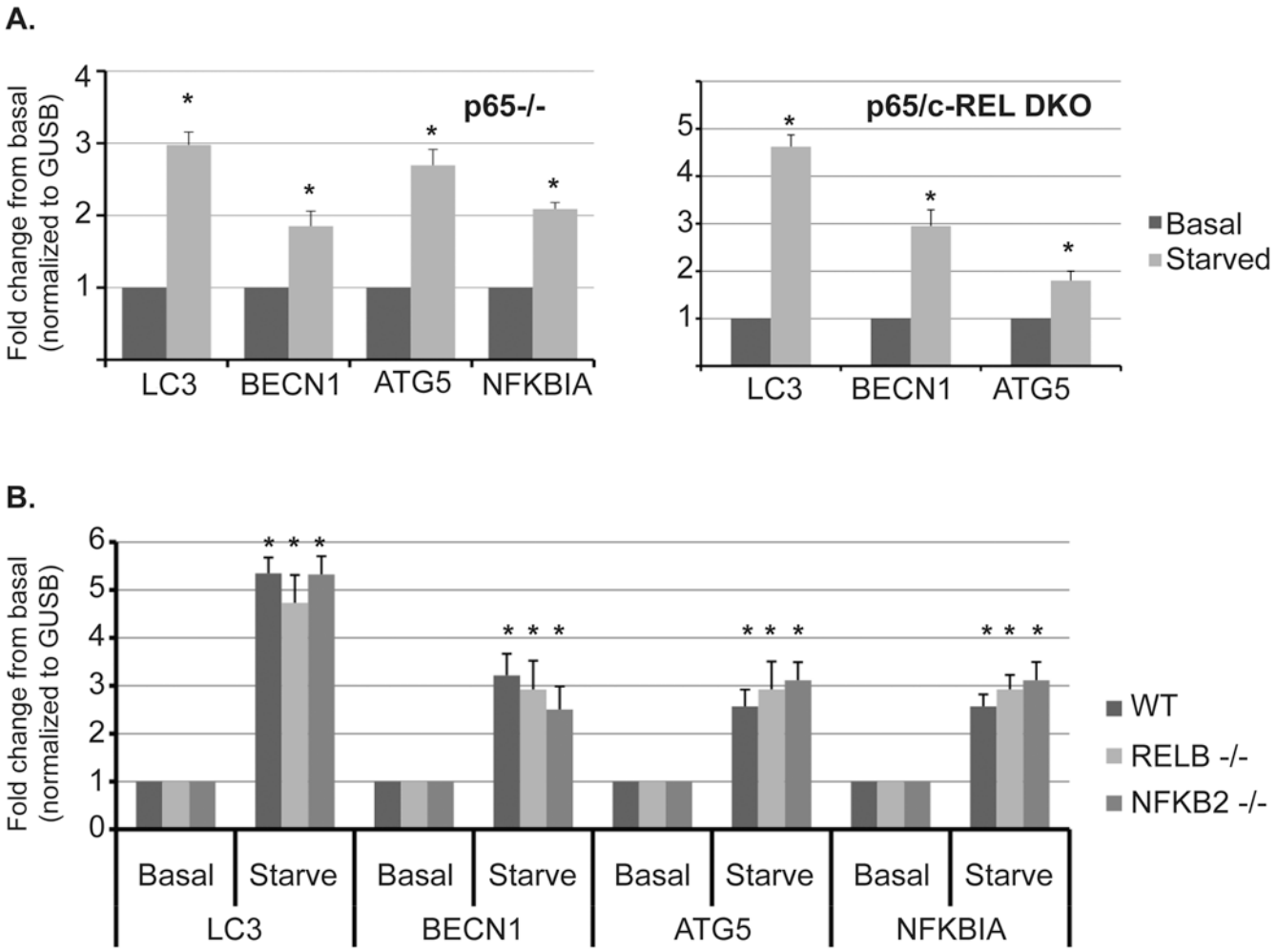


**Figure 3. Basal and starvation-induced autophagic gene expression is NF-κB-independent**

(**A**) p65 null (p65 -/-), p65/c-Rel double null (p65/c-Rel DKO) cells were grown in basal or starvation media for 18 hours and pro-autophagic gene expression was measured. (**B**) WT, RelB, and NFKB2 null (RelB-/-, NFKB2-/-) mEFs were grown in basal or starvation media for 12 hours and pro-autophagic gene expression was measured. Data is represented as fold-change over basal expression, *<.05.